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[Continued on next page]

(54) Title: MOLECULES FOR DIAGNOSTICS AND THERAPEUTICS

(57) Abstract: The present invention provides purified human polynucleotides for diagnostics and therapeutics (dithp). Also encompassed are the polypeptides (DITHP) encoded by dithp. The invention also provides for the use of dithp, or complements, oligonucleotides, or fragments thereof in diagnostic assays. The invention further provides for vectors and host cells containing dithp for the expression of DITHP. The invention additionally provides for the use of isolated and purified DITHP to induce antibodies and to screen libraries of compounds and the use of anti-DITHP antibodies in diagnostic assays. Also provided are microarrays containing dithp and methods of use.

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## MOLECULES FOR DIAGNOSTICS AND THERAPEUTICS

### TECHNICAL FIELD

The present invention relates to human molecules for diagnostics and therapeutics and to the use of these sequences in the diagnosis, study, prevention, and treatment of diseases associated with human molecules.

### BACKGROUND OF THE INVENTION

The human genome is comprised of thousands of genes, many encoding gene products that function in the maintenance and growth of the various cells and tissues in the body. Aberrant expression or mutations in these genes and their products is the cause of, or is associated with, a variety of human diseases such as cancer and other cell proliferative disorders, autoimmune/inflammatory disorders, infections, developmental disorders, endocrine disorders, metabolic disorders, neurological disorders, gastrointestinal disorders, transport disorders, and connective tissue disorders. The identification of these genes and their products is the basis of an ever-expanding effort to find markers for early detection of diseases, and targets for their prevention and treatment. Therefore, these genes and their products are useful as diagnostics and therapeutics. These genes may encode, for example, enzyme molecules, molecules associated with growth and development, biochemical pathway molecules, extracellular information transmission molecules, receptor molecules, intracellular signaling molecules, membrane transport molecules, protein modification and maintenance molecules, nucleic acid synthesis and modification molecules, adhesion molecules, antigen recognition molecules, secreted and extracellular matrix molecules, cytoskeletal molecules, ribosomal molecules, electron transfer associated molecules, transcription factor molecules, chromatin molecules, cell membrane molecules, and organelle associated molecules.

For example, cancer represents a type of cell proliferative disorder that affects nearly every tissue in the body. A wide variety of molecules, either aberrantly expressed or mutated, can be the cause of, or involved with, various cancers because tissue growth involves complex and ordered patterns of cell proliferation, cell differentiation, and apoptosis. Cell proliferation must be regulated to maintain both the number of cells and their spatial organization. This regulation depends upon the appropriate expression of proteins which control cell cycle progression in response to extracellular signals such as growth factors and other mitogens, and intracellular cues such as DNA damage or nutrient starvation. Molecules which directly or indirectly modulate cell cycle progression fall into several categories, including growth factors and their receptors, second messenger and signal transduction proteins, oncogene products, tumor-suppressor proteins, and mitosis-promoting factors. Aberrant expression or mutations in any of these gene products can result in cell proliferative disorders

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such as cancer. Oncogenes are genes generally derived from normal genes that, through abnormal expression or mutation, can effect the transformation of a normal cell to a malignant one (oncogenesis). Oncoproteins, encoded by oncogenes, can affect cell proliferation in a variety of ways and include growth factors, growth factor receptors, intracellular signal transducers, nuclear transcription factors, and cell-cycle control proteins. In contrast, tumor-suppressor genes are involved in inhibiting cell proliferation. Mutations which cause reduced function or loss of function in tumor-suppressor genes result in aberrant cell proliferation and cancer. Although many different genes and their products have been found to be associated with cell proliferative disorders such as cancer, many more may exist that are yet to be discovered.

DNA-based arrays can provide a simple way to explore the expression of a single polymorphic gene or a large number of genes. When the expression of a single gene is explored, DNA-based arrays are employed to detect the expression of specific gene variants. For example, a p53 tumor suppressor gene array is used to determine whether individuals are carrying mutations that predispose them to cancer. A cytochrome p450 gene array is useful to determine whether individuals have one of a number of specific mutations that could result in increased drug metabolism, drug resistance or drug toxicity.

DNA-based array technology is especially relevant for the rapid screening of expression of a large number of genes. There is a growing awareness that gene expression is affected in a global fashion. A genetic predisposition, disease or therapeutic treatment may affect, directly or indirectly, the expression of a large number of genes. In some cases the interactions may be expected, such as when the genes are part of the same signaling pathway. In other cases, such as when the genes participate in separate signaling pathways, the interactions may be totally unexpected. Therefore, DNA-based arrays can be used to investigate how genetic predisposition, disease, or therapeutic treatment affects the expression of a large number of genes.

## Enzyme Molecules

SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5 encode, for example, human enzyme molecules.

The cellular processes of biogenesis and biodegradation involve a number of key enzyme classes including oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. These enzyme classes are each comprised of numerous substrate-specific enzymes having precise and well regulated functions. These enzymes function by facilitating metabolic processes such as glycolysis, the tricarboxylic cycle, and fatty acid metabolism; synthesis or degradation of amino acids, steroids, phospholipids, alcohols, etc.; regulation of cell signalling, proliferation, inflammation, apoptosis, etc., and through catalyzing critical steps in DNA replication and repair, and the process of translation.

### Oxidoreductases



Many pathways of biogenesis and biodegradation require oxidoreductase (dehydrogenase or reductase) activity, coupled to the reduction or oxidation of a donor or acceptor cofactor. Potential cofactors include cytochromes, oxygen, disulfide, iron-sulfur proteins, flavin adenine dinucleotide (FAD), and the nicotinamide adenine dinucleotides NAD and NADP (Newsholme, E.A. and A.R. Leech (1983) Biochemistry for the Medical Sciences, John Wiley and Sons, Chichester, U.K., pp. 779-793). Reductase activity catalyzes the transfer of electrons between substrate(s) and cofactor(s) with concurrent oxidation of the cofactor. The reverse dehydrogenase reaction catalyzes the reduction of a cofactor and consequent oxidation of the substrate. Oxidoreductase enzymes are a broad superfamily of proteins that catalyze numerous reactions in all cells of organisms ranging from bacteria to plants to humans. These reactions include metabolism of sugar, certain detoxification reactions in the liver, and the synthesis or degradation of fatty acids, amino acids, glucocorticoids, estrogens, androgens, and prostaglandins. Different family members are named according to the direction in which their reactions are typically catalyzed; thus they may be referred to as oxidoreductases, oxidases, reductases, or dehydrogenases. In addition, family members often have distinct cellular localizations, including the cytosol, the plasma membrane, mitochondrial inner or outer membrane, and peroxisomes.

Short-chain alcohol dehydrogenases (SCADs) are a family of dehydrogenases that only share 15% to 30% sequence identity, with similarity predominantly in the coenzyme binding domain and the substrate binding domain. In addition to the well-known role in detoxification of ethanol, SCADs are also involved in synthesis and degradation of fatty acids, steroids, and some prostaglandins, and are therefore implicated in a variety of disorders such as lipid storage disease, myopathy, SCAD deficiency, and certain genetic disorders. For example, retinol dehydrogenase is a SCAD-family member (Simon, A. et al. (1995) *J. Biol. Chem.* 270:1107-1112) that converts retinol to retinal, the precursor of retinoic acid. Retinoic acid, a regulator of differentiation and apoptosis, has been shown to down-regulate genes involved in cell proliferation and inflammation (Chai, X. et al. (1995) *J. Biol. Chem.* 270:3900-3904). In addition, retinol dehydrogenase has been linked to hereditary eye diseases such as autosomal recessive childhood-onset severe retinal dystrophy (Simon, A. et al. (1996) *Genomics* 36:424-430).

Propagation of nerve impulses, modulation of cell proliferation and differentiation, induction of the immune response, and tissue homeostasis involve neurotransmitter metabolism (Weiss, B. (1991) *Neurotoxicology* 12:379-386; Collins, S.M. et al. (1992) *Ann. N.Y. Acad. Sci.* 664:415-424; Brown, J.K. and H. Imam (1991) *J. Inherit. Metab. Dis.* 14:436-458). Many pathways of neurotransmitter metabolism require oxidoreductase activity, coupled to reduction or oxidation of a cofactor, such as NAD<sup>+</sup>/NADH (Newsholme, E.A. and A.R. Leech (1983) Biochemistry for the Medical Sciences, John Wiley and Sons, Chichester, U.K. pp. 779-793). Degradation of

catecholamines (epinephrine or norepinephrine) requires alcohol dehydrogenase (in the brain) or aldehyde dehydrogenase (in peripheral tissue). NAD<sup>+</sup>-dependent aldehyde dehydrogenase oxidizes 5-hydroxyindole-3-acetate (the product of 5-hydroxytryptamine (serotonin) metabolism) in the brain, blood platelets, liver and pulmonary endothelium (Newsholme, supra, p. 786). Other neurotransmitter degradation pathways that utilize NAD<sup>+</sup>/NADH-dependent oxidoreductase activity include those of L-DOPA (precursor of dopamine, a neuronal excitatory compound), glycine (an inhibitory neurotransmitter in the brain and spinal cord), histamine (liberated from mast cells during the inflammatory response), and taurine (an inhibitory neurotransmitter of the brain stem, spinal cord and retina) (Newsholme, supra, pp. 790, 792). Epigenetic or genetic defects in neurotransmitter metabolic pathways can result in a spectrum of disease states in different tissues including Parkinson disease and inherited myoclonus (McCannc, K.L. and S.E. Huether (1994) Pathophysiology, Mosby-Year Book, Inc., St. Louis MO, pp. 402-404; Gundlach, A.L. (1990) FASEB J. 4:2761-2766).

Tetrahydrofolate is a derivatized glutamate molecule that acts as a carrier, providing activated one-carbon units to a wide variety of biosynthetic reactions, including synthesis of purines, pyrimidines, and the amino acid methionine. Tetrahydrofolate is generated by the activity of a holoenzyme complex called tetrahydrofolate synthase, which includes three enzyme activities: tetrahydrofolate dehydrogenase, tetrahydrofolate cyclohydrolase, and tetrahydrofolate synthetase. Thus, tetrahydrofolate dehydrogenase plays an important role in generating building blocks for nucleic and amino acids, crucial to proliferating cells.

3-Hydroxyacyl-CoA dehydrogenase (3HACD) is involved in fatty acid metabolism. It catalyzes the reduction of 3-hydroxyacyl-CoA to 3-oxoacyl-CoA, with concomitant oxidation of NAD to NADH, in the mitochondria and peroxisomes of eukaryotic cells. In peroxisomes, 3HACD and enoyl-CoA hydratase form an enzyme complex called bifunctional enzyme, defects in which are associated with peroxisomal bifunctional enzyme deficiency. This interruption in fatty acid metabolism produces accumulation of very-long chain fatty acids, disrupting development of the brain, bone, and adrenal glands. Infants born with this deficiency typically die within 6 months (Watkins, P. et al. (1989) *J. Clin. Invest.* 83:771-777; Online Mendelian Inheritance in Man (OMIM), #261515). The neurodegeneration that is characteristic of Alzheimer's disease involves development of extracellular plaques in certain brain regions. A major protein component of these plaques is the peptide amyloid- $\beta$  (A $\beta$ ), which is one of several cleavage products of amyloid precursor protein (APP). 3HACD has been shown to bind the A $\beta$  peptide, and is overexpressed in neurons affected in Alzheimer's disease. In addition, an antibody against 3HACD can block the toxic effects of A $\beta$  in a cell culture model of Alzheimer's disease (Yan, S. et al. (1997) *Nature* 389:689-695; OMIM, #602057).

Steroids, such as estrogen, testosterone, corticosterone, and others, are generated from a

common precursor, cholesterol, and are interconverted into one another. A wide variety of enzymes act upon cholesterol, including a number of dehydrogenases. Steroid dehydrogenases, such as the hydroxysteroid dehydrogenases, are involved in hypertension, fertility, and cancer (Duax, W.L. and D. Ghosh (1997) *Steroids* 62:95-100). One such dehydrogenase is 3-oxo-5- $\alpha$ -steroid dehydrogenase (OASD), a microsomal membrane protein highly expressed in prostate and other androgen-responsive tissues. OASD catalyzes the conversion of testosterone into dihydrotestosterone, which is the most potent androgen. Dihydrotestosterone is essential for the formation of the male phenotype during embryogenesis, as well as for proper androgen-mediated growth of tissues such as the prostate and male genitalia. A defect in OASD that prevents the conversion of testosterone into dihydrotestosterone leads to a rare form of male pseudohermaphroditis, characterized by defective formation of the external genitalia (Andersson, S. et al. (1991) *Nature* 354:159-161; Labrie, F. et al. (1992) *Endocrinology* 131:1571-1573; OMIM #264600). Thus, OASD plays a central role in sexual differentiation and androgen physiology.

17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ HSD6) plays an important role in the regulation of the male reproductive hormone, dihydrotestosterone (DHTT). 17 $\beta$ HSD6 acts to reduce levels of DHTT by oxidizing a precursor of DHTT, 3 $\alpha$ -diol, to androsterone which is readily glucuronidated and removed from tissues. 17 $\beta$ HSD6 is active with both androgen and estrogen substrates when expressed in embryonic kidney 293 cells. At least five other isozymes of 17 $\beta$ HSD have been identified that catalyze oxidation and/or reduction reactions in various tissues with preferences for different steroid substrates (Biswas, M.G. and D.W. Russell (1997) *J. Biol. Chem.* 272:15959-15966). For example, 17 $\beta$ HSD1 preferentially reduces estradiol and is abundant in the ovary and placenta. 17 $\beta$ HSD2 catalyzes oxidation of androgens and is present in the endometrium and placenta. 17 $\beta$ HSD3 is exclusively a reductive enzyme in the testis (Geissler, W.M. et al. (1994) *Nat. Genet.* 7:34-39). An excess of androgens such as DHTT can contribute to certain disease states such as benign prostatic hyperplasia and prostate cancer.

Oxidoreductases are components of the fatty acid metabolism pathways in mitochondria and peroxisomes. The main beta-oxidation pathway degrades both saturated and unsaturated fatty acids, while the auxiliary pathway performs additional steps required for the degradation of unsaturated fatty acids. The auxiliary beta-oxidation enzyme 2,4-dienoyl-CoA reductase catalyzes the removal of even-numbered double bonds from unsaturated fatty acids prior to their entry into the main beta-oxidation pathway. The enzyme may also remove odd-numbered double bonds from unsaturated fatty acids (Koivuranta, K.T. et al. (1994) *Biochem. J.* 304:787-792; Smeland, T.E. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:6673-6677). 2,4-dienoyl-CoA reductase is located in both mitochondria and peroxisomes. Inherited deficiencies in mitochondrial and peroxisomal beta-oxidation enzymes are associated with severe diseases, some of which manifest themselves soon after birth and lead to death

within a few years. Defects in beta-oxidation are associated with Reye's syndrome, Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum's disease, acyl-CoA oxidase deficiency, and bifunctional protein deficiency (Suzuki, Y. et al. (1994) *Am. J. Hum. Genet.* 54:36-43; Hoefler, supra; Cotran, R.S. et al. (1994) Robbins Pathologic Basis of Disease, W.B. Saunders Co., Philadelphia PA, p.866). Peroxisomal beta-oxidation is impaired in cancerous tissue. Although neoplastic human breast epithelial cells have the same number of peroxisomes as do normal cells, fatty acyl-CoA oxidase activity is lower than in control tissue (el Bouhtoury, F. et al. (1992) *J. Pathol.* 166:27-35). Human colon carcinomas have fewer peroxisomes than normal colon tissue and have lower fatty-acyl-CoA oxidase and bifunctional enzyme (including enoyl-CoA hydratase) activities than normal tissue (Cable, S. et al. (1992) *Virchows Arch. B Cell Pathol. Incl. Mol. Pathol.* 62:221-226). Another important oxidoreductase is isocitrate dehydrogenase, which catalyzes the conversion of isocitrate to  $\alpha$ -ketoglutarate, a substrate of the citric acid cycle. Isocitrate dehydrogenase can be either NAD or NADP dependent, and is found in the cytosol, mitochondria, and peroxisomes. Activity of isocitrate dehydrogenase is regulated developmentally, and by hormones, neurotransmitters, and growth factors.

Hydroxypyruvate reductase (HPR), a peroxisomal 2-hydroxyacid dehydrogenase in the glycolate pathway, catalyzes the conversion of hydroxypyruvate to glycerate with the oxidation of both NADH and NADPH. The reverse dehydrogenase reaction reduces  $\text{NAD}^+$  and  $\text{NADP}^+$ . HPR recycles nucleotides and bases back into pathways leading to the synthesis of ATP and GTP. ATP and GTP are used to produce DNA and RNA and to control various aspects of signal transduction and energy metabolism. Inhibitors of purine nucleotide biosynthesis have long been employed as antiproliferative agents to treat cancer and viral diseases. HPR also regulates biochemical synthesis of serine and cellular serine levels available for protein synthesis.

The mitochondrial electron transport (or respiratory) chain is a series of oxidoreductase-type enzyme complexes in the mitochondrial membrane that is responsible for the transport of electrons from NADH through a series of redox centers within these complexes to oxygen, and the coupling of this oxidation to the synthesis of ATP (oxidative phosphorylation). ATP then provides the primary source of energy for driving a cell's many energy-requiring reactions. The key complexes in the respiratory chain are NADH:ubiquinone oxidoreductase (complex I), succinate:ubiquinone oxidoreductase (complex II), cytochrome  $c_1$ -b oxidoreductase (complex III), cytochrome c oxidase (complex IV), and ATP synthase (complex V) (Alberts, B. et al. (1994) Molecular Biology of the Cell, Garland Publishing, Inc., New York NY, pp. 677-678). All of these complexes are located on the inner matrix side of the mitochondrial membrane except complex II, which is on the cytosolic side. Complex II transports electrons generated in the citric acid cycle to the respiratory chain. The electrons generated by oxidation of succinate to fumarate in the citric acid cycle are transferred

through electron carriers in complex II to membrane bound ubiquinone (Q). Transcriptional regulation of these nuclear-encoded genes appears to be the predominant means for controlling the biogenesis of respiratory enzymes. Defects and altered expression of enzymes in the respiratory chain are associated with a variety of disease conditions.

5 Other dehydrogenase activities using NAD as a cofactor are also important in mitochondrial function. 3-hydroxyisobutyrate dehydrogenase (3HBD), important in valine catabolism, catalyzes the NAD-dependent oxidation of 3-hydroxyisobutyrate to methylmalonate semialdehyde within mitochondria. Elevated levels of 3-hydroxyisobutyrate have been reported in a number of disease states, including ketoacidosis, methylmalonic acidemia, and other disorders associated with  
10 deficiencies in methylmalonate semialdehyde dehydrogenase (Rougraff, P.M. et al. (1989) J. Biol. Chem. 264:5899-5903).

Another mitochondrial dehydrogenase important in amino acid metabolism is the enzyme isovaleryl-CoA-dehydrogenase (IVD). IVD is involved in leucine metabolism and catalyzes the oxidation of isovaleryl-CoA to 3-methylcrotonyl-CoA. Human IVD is a tetrameric flavoprotein that  
15 is encoded in the nucleus and synthesized in the cytosol as a 45 kDa precursor with a mitochondrial import signal sequence. A genetic deficiency, caused by a mutation in the gene encoding IVD, results in the condition known as isovaleric acidemia. This mutation results in inefficient mitochondrial import and processing of the IVD precursor (Vockley, J. et al. (1992) J. Biol. Chem. 267:2494-2501).

#### Transferases

20 Transferases are enzymes that catalyze the transfer of molecular groups. The reaction may involve an oxidation, reduction, or cleavage of covalent bonds, and is often specific to a substrate or to particular sites on a type of substrate. Transferases participate in reactions essential to such functions as synthesis and degradation of cell components, regulation of cell functions including cell signaling, cell proliferation, inflammation, apoptosis, secretion and excretion. Transferases are  
25 involved in key steps in disease processes involving these functions. Transferases are frequently classified according to the type of group transferred. For example, methyl transferases transfer one-carbon methyl groups, amino transferases transfer nitrogenous amino groups, and similarly denominated enzymes transfer aldehyde or ketone, acyl, glycosyl, alkyl or aryl, isoprenyl, saccharyl, phosphorous-containing, sulfur-containing, or selenium-containing groups, as well as small  
30 enzymatic groups such as Coenzyme A.

Acyl transferases include peroxisomal carnitine octanoyl transferase, which is involved in the fatty acid beta-oxidation pathway, and mitochondrial carnitine palmitoyl transferases, involved in fatty acid metabolism and transport. Choline O-acetyl transferase catalyzes the biosynthesis of the neurotransmitter acetylcholine.

35 Amino transferases play key roles in protein synthesis and degradation, and they contribute to

other processes as well. For example, the amino transferase 5-aminolevulinic acid synthase catalyzes the addition of succinyl-CoA to glycine, the first step in heme biosynthesis. Other amino transferases participate in pathways important for neurological function and metabolism. For example, glutamine-phenylpyruvate amino transferase, also known as glutamine transaminase K (GTK), catalyzes several reactions with a pyridoxal phosphate cofactor. GTK catalyzes the reversible conversion of L-glutamine and phenylpyruvate to 2-oxoglutarate and L-phenylalanine. Other amino acid substrates for GTK include L-methionine, L-histidine, and L-tyrosine. GTK also catalyzes the conversion of kynurenine to kynurenic acid, a tryptophan metabolite that is an antagonist of the N-methyl-D-aspartate (NMDA) receptor in the brain and may exert a neuromodulatory function. Alteration of the kynurenine metabolic pathway may be associated with several neurological disorders. GTK also plays a role in the metabolism of halogenated xenobiotics conjugated to glutathione, leading to nephrotoxicity in rats and neurotoxicity in humans. GTK is expressed in kidney, liver, and brain. Both human and rat GTKs contain a putative pyridoxal phosphate binding site (ExPASy ENZYME: EC 2.6.1.64; Perry, S.J. et al. (1993) *Mol. Pharmacol.* 43:660-665; Perry, S. et al. (1995) *FEBS Lett.* 360:277-280; and Alberati-Giani, D. et al. (1995) *J. Neurochem.* 64:1448-1455). A second amino transferase associated with this pathway is kynurenine/ $\alpha$ -aminoadipate amino transferase (AadAT). AadAT catalyzes the reversible conversion of  $\alpha$ -aminoadipate and  $\alpha$ -ketoglutarate to  $\alpha$ -ketoadipate and L-glutamate during lysine metabolism. AadAT also catalyzes the transamination of kynurenine to kynurenic acid. A cytosolic AadAT is expressed in rat kidney, liver, and brain (Nakatani, Y. et al. (1970) *Biochim. Biophys. Acta* 198:219-228; Buchli, R. et al. (1995) *J. Biol. Chem.* 270:29330-29335).

Glycosyl transferases include the mammalian UDP-glucuronosyl transferases, a family of membrane-bound microsomal enzymes catalyzing the transfer of glucuronic acid to lipophilic substrates in reactions that play important roles in detoxification and excretion of drugs, carcinogens, and other foreign substances. Another mammalian glycosyl transferase, mammalian UDP-galactose-ceramide galactosyl transferase, catalyzes the transfer of galactose to ceramide in the synthesis of galactocerebrosides in myelin membranes of the nervous system. The UDP-glycosyl transferases share a conserved signature domain of about 50 amino acid residues (PROSITE: PDOC00359, <http://expasy.hcuge.ch/sprot/prosite.html>).

Methyl transferases are involved in a variety of pharmacologically important processes. Nicotinamide N-methyl transferase catalyzes the N-methylation of nicotinamides and other pyridines, an important step in the cellular handling of drugs and other foreign compounds. Phenylethanolamine N-methyl transferase catalyzes the conversion of noradrenaline to adrenaline. 6-O-methylguanine-DNA methyl transferase reverses DNA methylation, an important step in carcinogenesis. Uroporphyrin-III C-methyl transferase, which catalyzes the transfer of two methyl

groups from S-adenosyl-L-methionine to uroporphyrinogen **III**, is the first specific enzyme in the biosynthesis of cobalamin, a dietary enzyme whose uptake is deficient in pernicious anemia. Protein-arginine methyl transferases catalyze the posttranslational methylation of arginine residues in proteins, resulting in the mono- and dimethylation of arginine on the guanidino group. Substrates include histones, myelin basic protein, and heterogeneous nuclear ribonucleoproteins involved in mRNA processing, splicing, and transport. Protein-arginine methyl transferase interacts with proteins upregulated by mitogens, with proteins involved in chronic lymphocytic leukemia, and with interferon, suggesting an important role for methylation in cytokine receptor signaling (Lin, W.-J. et al. (1996) *J. Biol. Chem.* 271:15034-15044; Abramovich, C. et al. (1997) *EMBO J.* 16:260-266; and Scott, H.S. et al. (1998) *Genomics* 48:330-340).

Phosphotransferases catalyze the transfer of high-energy phosphate groups and are important in energy-requiring and -releasing reactions. The metabolic enzyme creatine kinase catalyzes the reversible phosphate transfer between creatine/creatine phosphate and ATP/ADP. Glycocyamine kinase catalyzes phosphate transfer from ATP to guanidoacetate, and arginine kinase catalyzes phosphate transfer from ATP to arginine. A cysteine-containing active site is conserved in this family (PROSITE: PDOC00103).

Prenyl transferases are heterodimers, consisting of an alpha and a beta subunit, that catalyze the transfer of an isoprenyl group. An example of a prenyl transferase is the mammalian protein farnesyl transferase. The alpha subunit of farnesyl transferase consists of 5 repeats of 34 amino acids each, with each repeat containing an invariant tryptophan (PROSITE: PDOC00703).

Saccharyl transferases are glycosylating enzymes involved in a variety of metabolic processes. Oligosaccharyl transferase-48, for example, is a receptor for advanced glycation endproducts. Accumulation of these endproducts is observed in vascular complications of diabetes, macrovascular disease, renal insufficiency, and Alzheimer's disease (Thornalley, P.J. (1998) *Cell Mol. Biol. (Noisy-Le-Grand)* 44:1013-1023).

Coenzyme A (CoA) transferase catalyzes the transfer of CoA between two carboxylic acids. Succinyl CoA:3-oxoacid CoA transferase, for example, transfers CoA from succinyl-CoA to a recipient such as acetoacetate. Acetoacetate is essential to the metabolism of ketone bodies, which accumulate in tissues affected by metabolic disorders such as diabetes (PROSITE: PDOC00980).

### 30 Hydrolases

Hydrolysis is the breaking of a covalent bond in a substrate by introduction of a molecule of water. The reaction involves a nucleophilic attack by the water molecule's oxygen atom on a target bond in the substrate. The water molecule is split across the target bond, breaking the bond and generating two product molecules. Hydrolases participate in reactions essential to such functions as synthesis and degradation of cell components, and for regulation of cell functions including cell

signaling, cell proliferation, inflammation, apoptosis, secretion and excretion. Hydrolases are involved in key steps in disease processes involving these functions. Hydrolytic enzymes, or hydrolases, may be grouped by substrate specificity into classes including phosphatases, peptidases, lysophospholipases, phosphodiesterases, glycosidases, and glyoxalases.

5        Phosphatases hydrolytically remove phosphate groups from proteins, an energy-providing step that regulates many cellular processes, including intracellular signaling pathways that in turn control cell growth and differentiation, cell-cell contact, the cell cycle, and oncogenesis.

      Lysophospholipases (LPLs) regulate intracellular lipids by catalyzing the hydrolysis of ester bonds to remove an acyl group, a key step in lipid degradation. Small LPL isoforms, approximately  
10    15-30 kD, function as hydrolases; larger isoforms function both as hydrolases and transacylases. A particular substrate for LPLs, lysophosphatidylcholine, causes lysis of cell membranes. LPL activity is regulated by signaling molecules important in numerous pathways, including the inflammatory response.

      Peptidases, also called proteases, cleave peptide bonds that form the backbone of peptide or  
15    protein chains. Proteolytic processing is essential to cell growth, differentiation, remodeling, and homeostasis as well as inflammation and immune response. Since typical protein half-lives range from hours to a few days, peptidases are continually cleaving precursor proteins to their active form, removing signal sequences from targeted proteins, and degrading aged or defective proteins. Peptidases function in bacterial, parasitic, and viral invasion and replication within a host. Examples  
20    of peptidases include trypsin and chymotrypsin (components of the complement cascade and the blood-clotting cascade) lysosomal cathepsins, calpains, pepsin, renin, and chymosin (Beynon, R.J. and J.S. Bond (1994) Proteolytic Enzymes: A Practical Approach, Oxford University Press, New York NY, pp. 1-5).

      The phosphodiesterases catalyze the hydrolysis of one of the two ester bonds in a  
25    phosphodiester compound. Phosphodiesterases are therefore crucial to a variety of cellular processes. Phosphodiesterases include DNA and RNA endo- and exo-nucleases, which are essential to cell growth and replication as well as protein synthesis. Another phosphodiesterase is acid sphingomyelinase, which hydrolyzes the membrane phospholipid sphingomyelin to ceramide and phosphorylcholine. Phosphorylcholine is used in the synthesis of phosphatidylcholine, which is  
30    involved in numerous intracellular signaling pathways. Ceramide is an essential precursor for the generation of gangliosides, membrane lipids found in high concentration in neural tissue. Defective acid sphingomyelinase phosphodiesterase leads to a build-up of sphingomyelin molecules in lysosomes, resulting in Niemann-Pick disease.

      Glycosidases catalyze the cleavage of hemiacetyl bonds of glycosides, which are compounds  
35    that contain one or more sugar. Mammalian lactase-phlorizin hydrolase, for example, is an intestinal



enzyme that splits lactose. Mammalian beta-galactosidase removes the terminal galactose from gangliosides, glycoproteins, and glycosaminoglycans, and deficiency of this enzyme is associated with a gangliosidosis known as Morquio disease type B. Vertebrate lysosomal alpha-glucosidase, which hydrolyzes glycogen, maltose, and isomaltose, and vertebrate intestinal sucrase-isomaltase, which hydrolyzes sucrose, maltose, and isomaltose, are widely distributed members of this family with highly conserved sequences at their active sites.

The glyoxylase system is involved in gluconeogenesis, the production of glucose from storage compounds in the body. It consists of glyoxylase I, which catalyzes the formation of S-D-lactoylglutathione from methylglyoxal, a side product of triose-phosphate energy metabolism, and glyoxylase II, which hydrolyzes S-D-lactoylglutathione to D-lactic acid and reduced glutathione. Glyoxylases are involved in hyperglycemia, non-insulin-dependent diabetes mellitus, the detoxification of bacterial toxins, and in the control of cell proliferation and microtubule assembly.

### Lyases

Lyases are a class of enzymes that catalyze the cleavage of C-C, C-O, C-N, C-S, C-(halide), P-O or other bonds without hydrolysis or oxidation to form two molecules, at least one of which contains a double bond (Stryer, L. (1995) Biochemistry W.H. Freeman and Co. New York, NY p.620). Lyases are critical components of cellular biochemistry with roles in metabolic energy production including fatty acid metabolism, as well as other diverse enzymatic processes. Further classification of lyases reflects the type of bond cleaved as well as the nature of the cleaved group.

The group of C-C lyases include carboxyl-lyases (decarboxylases), aldehyde-lyases (aldolases), oxo-acid-lyases and others. The C-O lyase group includes hydro-lyases, lyases acting on polysaccharides and other lyases. The C-N lyase group includes ammonia-lyases, amidine-lyases, amine-lyases (deaminases) and other lyases.

Proper regulation of lyases is critical to normal physiology. For example, mutation induced deficiencies in the uroporphyrinogen decarboxylase can lead to photosensitive cutaneous lesions in the genetically-linked disorder familial porphyria cutanea tarda (Mendez, M. et al. (1998) *Am. J. Genet.* 63:1363-1375). It has also been shown that adenosine deaminase (ADA) deficiency stems from genetic mutations in the ADA gene, resulting in the disorder severe combined immunodeficiency disease (SCID) (Hershtfeld, M.S. (1998) *Semin. Hematol.* 35:291-298).

### Isomerases

Isomerases are a class of enzymes that catalyze geometric or structural changes within a molecule to form a single product. This class includes racemases and epimerases, cis-trans-isomerases, intramolecular oxidoreductases, intramolecular transferases (mutases) and intramolecular lyases. Isomerases are critical components of cellular biochemistry with roles in metabolic energy production including glycolysis, as well as other diverse enzymatic processes (Stryer, L. (1995)

Racemases are a subset of isomerases that catalyze inversion of a molecule's configuration around the asymmetric carbon atom in a substrate having a single center of asymmetry, thereby interconverting two racemers. Epimerases are another subset of isomerases that catalyze inversion of configuration around an asymmetric carbon atom in a substrate with more than one center of symmetry, thereby interconverting two epimers. Racemases and epimerases can act on amino acids and derivatives, hydroxy acids and derivatives, as well as carbohydrates and derivatives. The interconversion of UDP-galactose and UDP-glucose is catalyzed by UDP-galactose-4'-epimerase. Proper regulation and function of this epimerase is essential to the synthesis of glycoproteins and glycolipids. Elevated blood galactose levels have been correlated with UDP-galactose-4'-epimerase deficiency in screening programs of infants (Gitzelmann, R. (1972) *Helv. Paediat. Acta* 27:125-130).

Another subgroup of isomerases are the transferases (or mutases). Transferases transfer a chemical group from one compound (the donor) to another compound (the acceptor). The types of groups transferred by these enzymes include acyl groups, amino groups, phosphate groups (phosphotransferases or phosphomutases), and others. The transferase carnitine palmitoyltransferase is an important component of fatty acid metabolism. Genetically-linked deficiencies in this transferase can lead to myopathy (Scriver, C.R. et al. (1995) The Metabolic and Molecular Basis of Inherited Disease. McGraw-Hill, New York NY, pp.1501-1533).

## Ligases

Ligases forming carbon-oxygen bonds include the aminoacyl-transfer RNA (tRNA)

synthetases which are important RNA-associated enzymes with roles in translation. Protein biosynthesis depends on each amino acid forming a linkage with the appropriate tRNA. The aminoacyl-tRNA synthetases are responsible for the activation and correct attachment of an amino acid with its cognate tRNA. The 20 aminoacyl-tRNA synthetase enzymes can be divided into two structural classes, and each class is characterized by a distinctive topology of the catalytic domain. Class I enzymes contain a catalytic domain based on the nucleotide-binding Rossman fold. Class II enzymes contain a central catalytic domain, which consists of a seven-stranded antiparallel  $\beta$ -sheet motif, as well as N- and C- terminal regulatory domains. Class II enzymes are separated into two groups based on the heterodimeric or homodimeric structure of the enzyme; the latter group is further subdivided by the structure of the N- and C-terminal regulatory domains (Hartlein, M. and S. Cusack (1995) J. Mol. Evol. 40:519-530). Autoantibodies against aminoacyl-tRNAs are generated by patients with dermatomyositis and polymyositis, and correlate strongly with complicating interstitial lung disease (ILD). These antibodies appear to be generated in response to viral infection, and coxsackie virus has been used to induce experimental viral myositis in animals.

Ligases forming carbon-sulfur bonds (Acid-thiol ligases) mediate a large number of cellular biosynthetic intermediary metabolism processes involve intermolecular transfer of carbon atom-containing substrates (carbon substrates). Examples of such reactions include the tricarboxylic acid cycle, synthesis of fatty acids and long-chain phospholipids, synthesis of alcohols and aldehydes, synthesis of intermediary metabolites, and reactions involved in the amino acid degradation pathways. Some of these reactions require input of energy, usually in the form of conversion of ATP to either ADP or AMP and pyrophosphate.

In many cases, a carbon substrate is derived from a small molecule containing at least two carbon atoms. The carbon substrate is often covalently bound to a larger molecule which acts as a carbon substrate carrier molecule within the cell. In the biosynthetic mechanisms described above, the carrier molecule is coenzyme A. Coenzyme A (CoA) is structurally related to derivatives of the nucleotide ADP and consists of 4'-phosphopantetheine linked via a phosphodiester bond to the alpha phosphate group of adenosine 3',5'-bisphosphate. The terminal thiol group of 4'-phosphopantetheine acts as the site for carbon substrate bond formation. The predominant carbon substrates which utilize CoA as a carrier molecule during biosynthesis and intermediary metabolism in the cell are acetyl, succinyl, and propionyl moieties, collectively referred to as acyl groups. Other carbon substrates include enoyl lipid, which acts as a fatty acid oxidation intermediate, and carnitine, which acts as an acetyl-CoA flux regulator/ mitochondrial acyl group transfer protein. Acyl-CoA and acetyl-CoA are synthesized in the cell by acyl-CoA synthetase and acetyl-CoA synthetase, respectively.

Activation of fatty acids is mediated by at least three forms of acyl-CoA synthetase activity:

i) acetyl-CoA synthetase, which activates acetate and several other low molecular weight carboxylic

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disorders such as cancer and infectious diseases.

Purine biosynthesis occurs de novo from the amino acids glycine and glutamine, and other small molecules. Three of the key reactions in this process are catalyzed by a trifunctional enzyme composed of glycinamide-ribonucleotide synthetase (GARS), aminoimidazole ribonucleotide  
 5 synthetase (AIRS), and glycinamide ribonucleotide transformylase (GART). Together these three enzymes combine ribosylamine phosphate with glycine to yield phosphoribosyl aminoimidazole, a precursor to both adenylate and guanylate nucleotides. This trifunctional protein has been implicated in the pathology of Down's syndrome (Aimi, J. et al. (1990) *Nucleic Acid Res.* 18:6665-6672). Adenylosuccinate synthetase catalyzes a later step in purine biosynthesis that converts inosinic acid to  
 10 adenylosuccinate, a key step on the path to ATP synthesis. This enzyme is also similar to another carbon-nitrogen ligase, argininosuccinate synthetase, that catalyzes a similar reaction in the urea cycle (Powell, S.M. et al. (1992) *FEBS Lett.* 303:4-10).

Like the de novo biosynthesis of purines, de novo synthesis of the pyrimidine nucleotides uridylylate and cytidylylate also arises from a common precursor, in this instance the nucleotide  
 15 orotidylylate derived from orotate and phosphoribosyl pyrophosphate (PPRP). Again a trifunctional enzyme comprising three carbon-nitrogen ligases plays a key role in the process. In this case the enzymes aspartate transcarbamylase (ATCase), carbamyl phosphate synthetase II, and dihydroorotase (DHOase) are encoded by a single gene called CAD. Together these three enzymes combine the initial reactants in pyrimidine biosynthesis, glutamine, CO<sub>2</sub> and ATP to form dihydroorotate, the  
 20 precursor to orotate and orotidylylate (Iwahana, H. et al. (1996) *Biochem. Biophys. Res. Commun.* 219:249-255). Further steps then lead to the synthesis of uridine nucleotides from orotidylylate. Cytidine nucleotides are derived from uridine-5'-triphosphate (UTP) by the amidation of UTP using glutamine as the amino donor and the enzyme CTP synthetase. Regulatory mutations in the human CTP synthetase are believed to confer multi-drug resistance to agents widely used in cancer therapy  
 25 (Yamauchi, M. et al. (1990) *EMBO J.* 9:2095-2099).

Ligases forming carbon-carbon bonds include the carboxylases acetyl-CoA carboxylase and pyruvate carboxylase. Acetyl-CoA carboxylase catalyzes the carboxylation of acetyl-CoA from CO<sub>2</sub> and H<sub>2</sub>O using the energy of ATP hydrolysis. Acetyl-CoA carboxylase is the rate-limiting step in the biogenesis of long-chain fatty acids. Two isoforms of acetyl-CoA carboxylase, types I and types II,  
 30 are expressed in human in a tissue-specific manner (Ha, J. et al. (1994) *Eur. J. Biochem.* 219:297-306). Pyruvate carboxylase is a nuclear-encoded mitochondrial enzyme that catalyzes the conversion of pyruvate to oxaloacetate, a key intermediate in the citric acid cycle.

Ligases forming phosphoric ester bonds include the DNA ligases involved in both DNA replication and repair. DNA ligases seal phosphodiester bonds between two adjacent nucleotides in a  
 35 DNA chain using the energy from ATP hydrolysis to first activate the free 5'-phosphate of one

nucleotide and then react it with the 3'-OH group of the adjacent nucleotide. This resealing reaction is used in both DNA replication to join small DNA fragments called Okazaki fragments that are transiently formed in the process of replicating new DNA, and in DNA repair. DNA repair is the process by which accidental base changes, such as those produced by oxidative damage, hydrolytic attack, or uncontrolled methylation of DNA, are corrected before replication or transcription of the DNA can occur. Bloom's syndrome is an inherited human disease in which individuals are partially deficient in DNA ligation and consequently have an increased incidence of cancer (Alberts, B. et al. (1994) The Molecular Biology of the Cell, Garland Publishing Inc., New York NY, p. 247).

#### 10 **Molecules Associated with Growth and Development**

SEQ ID NO:51 and SEQ ID NO:52 encode, for example, molecules associated with growth and development.

Human growth and development requires the spatial and temporal regulation of cell differentiation, cell proliferation, and apoptosis. These processes coordinately control reproduction, aging, embryogenesis, morphogenesis, organogenesis, and tissue repair and maintenance. At the cellular level, growth and development is governed by the cell's decision to enter into or exit from the cell division cycle and by the cell's commitment to a terminally differentiated state. These decisions are made by the cell in response to extracellular signals and other environmental cues it receives. The following discussion focuses on the molecular mechanisms of cell division, reproduction, cell differentiation and proliferation, apoptosis, and aging.

##### Cell Division

Cell division is the fundamental process by which all living things grow and reproduce. In unicellular organisms such as yeast and bacteria, each cell division doubles the number of organisms, while in multicellular species many rounds of cell division are required to replace cells lost by wear or by programmed cell death, and for cell differentiation to produce a new tissue or organ. Details of the cell division cycle may vary, but the basic process consists of three principle events. The first event, interphase, involves preparations for cell division, replication of the DNA, and production of essential proteins. In the second event, mitosis, the nuclear material is divided and separates to opposite sides of the cell. The final event, cytokinesis, is division and fission of the cell cytoplasm. The sequence and timing of cell cycle transitions is under the control of the cell cycle regulation system which controls the process by positive or negative regulatory circuits at various check points.

Regulated progression of the cell cycle depends on the integration of growth control pathways with the basic cell cycle machinery. Cell cycle regulators have been identified by selecting for human and yeast cDNAs that block or activate cell cycle arrest signals in the yeast mating pheromone pathway when they are overexpressed. Known regulators include human CPR (cell cycle progression

restoration) genes, such as CPR8 and CPR2, and yeast CDC (cell division control) genes, including CDC91, that block the arrest signals. The CPR genes express a variety of proteins including cyclins, tumor suppressor binding proteins, chaperones, transcription factors, translation factors, and RNA-binding proteins (Edwards, M.C. et al.(1997) Genetics 147:1063-1076).

5 Several cell cycle transitions, including the entry and exit of a cell from mitosis, are dependent upon the activation and inhibition of cyclin-dependent kinases (Cdk). The Cdk is composed of a kinase subunit, Cdk, and an activating subunit, cyclin, in a complex that is subject to many levels of regulation. There appears to be a single Cdk in Saccharomyces cerevisiae and Saccharomyces pombe whereas mammals have a variety of specialized Cdk. Cyclins act by binding to and activating  
10 cyclin-dependent protein kinases which then phosphorylate and activate selected proteins involved in the mitotic process. The Cdk-cyclin complex is both positively and negatively regulated by phosphorylation, and by targeted degradation involving molecules such as CDC4 and CDC53. In addition, Cdk is further regulated by binding to inhibitors and other proteins such as Suc1 that modify their specificity or accessibility to regulators (Patra, D. and W.G. Dunphy (1996) Genes Dev.  
15 10:1503-1515; and Mathias, N. et al. (1996) Mol. Cell Biol. 16:6634-6643).

#### Reproduction

The male and female reproductive systems are complex and involve many aspects of growth and development. The anatomy and physiology of the male and female reproductive systems are reviewed in (Guyton, A.C. (1991) Textbook of Medical Physiology, W.B. Saunders Co., Philadelphia  
20 PA, pp. 899-928).

The male reproductive system includes the process of spermatogenesis, in which the sperm are formed, and male reproductive functions are regulated by various hormones and their effects on accessory sexual organs, cellular metabolism, growth, and other bodily functions.

Spermatogenesis begins at puberty as a result of stimulation by gonadotropic hormones released from the anterior pituitary. Immature sperm (spermatogonia) undergo several mitotic cell divisions before undergoing meiosis and full maturation. The testes secrete several male sex hormones, the most abundant being testosterone, that is essential for growth and division of the immature sperm, and for the masculine characteristics of the male body. Three other male sex hormones, gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH) control  
30 sexual function.

The uterus, ovaries, fallopian tubes, vagina, and breasts comprise the female reproductive system. The ovaries and uterus are the source of ova and the location of fetal development, respectively. The fallopian tubes and vagina are accessory organs attached to the top and bottom of the uterus, respectively. Both the uterus and ovaries have additional roles in the development and loss of  
35 reproductive capability during a female's lifetime. The primary role of the breasts is lactation.

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Multiple endocrine signals from the ovaries, uterus, pituitary, hypothalamus, adrenal glands, and other tissues coordinate reproduction and lactation. These signals vary during the monthly menstruation cycle and during the female's lifetime. Similarly, the sensitivity of reproductive organs to these endocrine signals varies during the female's lifetime.

5       A combination of positive and negative feedback to the ovaries, pituitary and hypothalamus glands controls physiologic changes during the monthly ovulation and endometrial cycles. The anterior pituitary secretes two major gonadotropin hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), regulated by negative feedback of steroids, most notably by ovarian estradiol. If fertilization does not occur, estrogen and progesterone levels decrease. This sudden reduction of the  
10       ovarian hormones leads to menstruation, the desquamation of the endometrium.

Hormones further govern all the steps of pregnancy, parturition, lactation, and menopause. During pregnancy large quantities of human chorionic gonadotropin (hCG), estrogens, progesterone, and human chorionic somatomammotropin (hCS) are formed by the placenta. hCG, a glycoprotein similar to luteinizing hormone, stimulates the corpus luteum to continue producing more progesterone  
15       and estrogens, rather than to involute as occurs if the ovum is not fertilized. hCS is similar to growth hormone and is crucial for fetal nutrition.

The female breast also matures during pregnancy. Large amounts of estrogen secreted by the placenta trigger growth and branching of the breast milk ductal system while lactation is initiated by the secretion of prolactin by the pituitary gland.

20       Parturition involves several hormonal changes that increase uterine contractility toward the end of pregnancy, as follows. The levels of estrogens increase more than those of progesterone. Oxytocin is secreted by the neurohypophysis. Concomitantly, uterine sensitivity to oxytocin increases. The fetus itself secretes oxytocin, cortisol (from adrenal glands), and prostaglandins.

Menopause occurs when most of the ovarian follicles have degenerated. The ovary then  
25       produces less estradiol, reducing the negative feedback on the pituitary and hypothalamus glands. Mean levels of circulating FSH and LH increase, even as ovulatory cycles continue. Therefore, the ovary is less responsive to gonadotropins, and there is an increase in the time between menstrual cycles. Consequently, menstrual bleeding ceases and reproductive capability ends.

#### Cell Differentiation and Proliferation

30       Tissue growth involves complex and ordered patterns of cell proliferation, cell differentiation, and apoptosis. Cell proliferation must be regulated to maintain both the number of cells and their spatial organization. This regulation depends upon the appropriate expression of proteins which control cell cycle progression in response to extracellular signals, such as growth factors and other mitogens, and intracellular cues, such as DNA damage or nutrient starvation. Molecules which directly or  
35       indirectly modulate cell cycle progression fall into several categories, including growth factors and their



receptors, second messenger and signal transduction proteins, oncogene products, tumor-suppressor proteins, and mitosis-promoting factors.

Growth factors were originally described as serum factors required to promote cell proliferation. Most growth factors are large, secreted polypeptides that act on cells in their local environment. Growth factors bind to and activate specific cell surface receptors and initiate intracellular signal transduction cascades. Many growth factor receptors are classified as receptor tyrosine kinases which undergo autophosphorylation upon ligand binding. Autophosphorylation enables the receptor to interact with signal transduction proteins characterized by the presence of SH2 or SH3 domains (Src homology regions 2 or 3). These proteins then modulate the activity state of small G-proteins, such as Ras, Rab, and Rho, along with GTPase activating proteins (GAPs), guanine nucleotide releasing proteins (GNRPs), and other guanine nucleotide exchange factors. Small G proteins act as molecular switches that activate other downstream events, such as mitogen-activated protein kinase (MAP kinase) cascades. MAP kinases ultimately activate transcription of mitosis-promoting genes.

In addition to growth factors, small signaling peptides and hormones also influence cell proliferation. These molecules bind primarily to another class of receptor, the trimeric G-protein coupled receptor (GPCR), found predominantly on the surface of immune, neuronal and neuroendocrine cells. Upon ligand binding, the GPCR activates a trimeric G protein which in turn triggers increased levels of intracellular second messengers such as phospholipase C, Ca<sup>2+</sup>, and cyclic AMP. Most GPCR-mediated signaling pathways indirectly promote cell proliferation by causing the secretion or breakdown of other signaling molecules that have direct mitogenic effects. These signaling cascades often involve activation of kinases and phosphatases. Some growth factors, such as some members of the transforming growth factor beta (TGF- $\beta$ ) family, act on some cells to stimulate cell proliferation and on other cells to inhibit it. Growth factors may also stimulate a cell at one concentration and inhibit the same cell at another concentration. Most growth factors also have a multitude of other actions besides the regulation of cell growth and division: they can control the proliferation, survival, differentiation, migration, or function of cells depending on the circumstance. For example, the tumor necrosis factor/nerve growth factor (TNF/NGF) family can activate or inhibit cell death, as well as regulate proliferation and differentiation. The cell response depends on the type of cell, its stage of differentiation and transformation status, which surface receptors are stimulated, and the types of stimuli acting on the cell (Smith, A. et al. (1994) *Cell* 76:959-962; and Nocentini, G. et al. (1997) *Proc. Natl. Acad. Sci. USA* 94:6216-6221).

Neighboring cells in a tissue compete for growth factors, and when provided with "unlimited" quantities in a perfused system will grow to even higher cell densities before reaching density-dependent inhibition of cell division. Cells often demonstrate an anchorage dependence of cell division as well.

This anchorage dependence may be associated with the formation of focal contacts linking the cytoskeleton with the extracellular matrix (ECM). The expression of ECM components can be stimulated by growth factors. For example, TGF- $\beta$  stimulates fibroblasts to produce a variety of ECM proteins, including fibronectin, collagen, and tenascin (Pearson, C.A. et al. (1988) EMBO J. 7:2677-2981). In fact, for some cell types specific ECM molecules, such as laminin or fibronectin, may act as growth factors. Tenascin-C and -R, expressed in developing and lesioned neural tissue, provide stimulatory/anti-adhesive or inhibitory properties, respectively, for axonal growth (Faissner, A. (1997) Cell Tissue Res. 290:331-341).

Cancers are associated with the activation of oncogenes which are derived from normal cellular genes. These oncogenes encode oncoproteins which convert normal cells into malignant cells. Some oncoproteins are mutant isoforms of the normal protein, and other oncoproteins are abnormally expressed with respect to location or amount of expression. The latter category of oncoprotein causes cancer by altering transcriptional control of cell proliferation. Five classes of oncoproteins are known to affect cell cycle controls. These classes include growth factors, growth factor receptors, intracellular signal transducers, nuclear transcription factors, and cell-cycle control proteins. Viral oncogenes are integrated into the human genome after infection of human cells by certain viruses. Examples of viral oncogenes include v-src, v-abl, and v-fps.

Many oncogenes have been identified and characterized. These include sis, erbA, erbB, her-2, mutated  $G_s$ , src, abl, ras, crk, jun, fos, myc, and mutated tumor-suppressor genes such as RB, p53, mdm2, Cip1, p16, and cyclin D. Transformation of normal genes to oncogenes may also occur by chromosomal translocation. The Philadelphia chromosome, characteristic of chronic myeloid leukemia and a subset of acute lymphoblastic leukemias, results from a reciprocal translocation between chromosomes 9 and 22 that moves a truncated portion of the proto-oncogene c-abl to the breakpoint cluster region (bcr) on chromosome 22.

Tumor-suppressor genes are involved in regulating cell proliferation. Mutations which cause reduced or loss of function in tumor-suppressor genes result in uncontrolled cell proliferation. For example, the retinoblastoma gene product (RB), in a non-phosphorylated state, binds several early-response genes and suppresses their transcription, thus blocking cell division. Phosphorylation of RB causes it to dissociate from the genes, releasing the suppression, and allowing cell division to proceed.

### Apoptosis

Apoptosis is the genetically controlled process by which unneeded or defective cells undergo programmed cell death. Selective elimination of cells is as important for morphogenesis and tissue remodeling as is cell proliferation and differentiation. Lack of apoptosis may result in hyperplasia and other disorders associated with increased cell proliferation. Apoptosis is also a critical component of the immune response. Immune cells such as cytotoxic T-cells and natural killer cells prevent the spread

of disease by inducing apoptosis in tumor cells and virus-infected cells. In addition, immune cells that fail to distinguish self molecules from foreign molecules must be eliminated by apoptosis to avoid an autoimmune response.

Apoptotic cells undergo distinct morphological changes. Hallmarks of apoptosis include cell shrinkage, nuclear and cytoplasmic condensation, and alterations in plasma membrane topology. Biochemically, apoptotic cells are characterized by increased intracellular calcium concentration, fragmentation of chromosomal DNA, and expression of novel cell surface components.

The molecular mechanisms of apoptosis are highly conserved, and many of the key protein regulators and effectors of apoptosis have been identified. Apoptosis generally proceeds in response to a signal which is transduced intracellularly and results in altered patterns of gene expression and protein activity. Signaling molecules such as hormones and cytokines are known both to stimulate and to inhibit apoptosis through interactions with cell surface receptors. Transcription factors also play an important role in the onset of apoptosis. A number of downstream effector molecules, particularly proteases such as the cysteine proteases called caspases, have been implicated in the degradation of cellular components and the proteolytic activation of other apoptotic effectors.

#### Aging and Senescence

Studies of the aging process or senescence have shown a number of characteristic cellular and molecular changes (Fauci et al. (1998) Harrison's Principles of Internal Medicine, McGraw-Hill, New York NY, p.37). These characteristics include increases in chromosome structural abnormalities, DNA cross-linking, incidence of single-stranded breaks in DNA, losses in DNA methylation, and degradation of telomere regions. In addition to these DNA changes, post-translational alterations of proteins increase including, deamidation, oxidation, cross-linking, and nonenzymatic glycation. Still further molecular changes occur in the mitochondria of aging cells through deterioration of structure. These changes eventually contribute to decreased function in every organ of the body.

#### **Biochemical Pathway Molecules**

SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, and SEQ ID NO:50 encode, for example, biochemical pathway molecules.

Biochemical pathways are responsible for regulating metabolism, growth and development, protein secretion and trafficking, environmental responses, and ecological interactions including immune response and response to parasites.

#### DNA replication

Deoxyribonucleic acid (DNA), the genetic material, is found in both the nucleus and mitochondria of human cells. The bulk of human DNA is nuclear, in the form of linear chromosomes, while mitochondrial DNA is circular. DNA replication begins at specific sites called origins of

replication. Bidirectional synthesis occurs from the origin via two growing forks that move in opposite directions. Replication is semi-conservative, with each daughter duplex containing one old strand and its newly synthesized complementary partner. Proteins involved in DNA replication include DNA polymerases, DNA primase, telomerase, DNA helicase, topoisomerases, DNA ligases, replication factors, and DNA-binding proteins.

#### DNA Recombination and Repair

Cells are constantly faced with replication errors and environmental assault (such as ultraviolet irradiation) that can produce DNA damage. Damage to DNA consists of any change that modifies the structure of the molecule. Changes to DNA can be divided into two general classes, single base changes and structural distortions. Any damage to DNA can produce a mutation, and the mutation may produce a disorder, such as cancer.

Changes in DNA are recognized by repair systems within the cell. These repair systems act to correct the damage and thus prevent any deleterious effects of a mutational event. Repair systems can be divided into three general types, direct repair, excision repair, and retrieval systems. Proteins involved in DNA repair include DNA polymerase, excision repair proteins, excision and cross link repair proteins, recombination and repair proteins, RAD51 proteins, and BLN and WRN proteins that are homologs of RecQ helicase. When the repair systems are eliminated, cells become exceedingly sensitive to environmental mutagens, such as ultraviolet irradiation. Patients with disorders associated with a loss in DNA repair systems often exhibit a high sensitivity to environmental mutagens. Examples of such disorders include xeroderma pigmentosum (XP), Bloom's syndrome (BS), and Werner's syndrome (WS) (Yamagata, K. et al. (1998) Proc. Natl. Acad. Sci. USA 95:8733-8738), ataxia telangiectasia, Cockayne's syndrome, and Fanconi's anemia.

Recombination is the process whereby new DNA sequences are generated by the movements of large pieces of DNA. In homologous recombination, which occurs during meiosis and DNA repair, parent DNA duplexes align at regions of sequence similarity, and new DNA molecules form by the breakage and joining of homologous segments. Proteins involved include RAD51 recombinase. In site-specific recombination, two specific but not necessarily homologous DNA sequences are exchanged. In the immune system this process generates a diverse collection of antibody and T cell receptor genes. Proteins involved in site-specific recombination in the immune system include recombination activating genes 1 and 2 (RAG1 and RAG2). A defect in immune system site-specific recombination causes severe combined immunodeficiency disease in mice.

#### RNA Metabolism

Ribonucleic acid (RNA) is a linear single-stranded polymer of four nucleotides, ATP, CTP, UTP, and GTP. In most organisms, RNA is transcribed as a copy of DNA, the genetic material of the organism. In retroviruses RNA rather than DNA serves as the genetic material. RNA copies of the

genetic material encode proteins or serve various structural, catalytic, or regulatory roles in organisms. RNA is classified according to its cellular localization and function. Messenger RNAs (mRNAs) encode polypeptides. Ribosomal RNAs (rRNAs) are assembled, along with ribosomal proteins, into ribosomes, which are cytoplasmic particles that translate mRNA into polypeptides. Transfer RNAs (tRNAs) are cytosolic adaptor molecules that function in mRNA translation by recognizing both an mRNA codon and the amino acid that matches that codon. Heterogeneous nuclear RNAs (hnRNAs) include mRNA precursors and other nuclear RNAs of various sizes. Small nuclear RNAs (snRNAs) are a part of the nuclear spliceosome complex that removes intervening, non-coding sequences (introns) and rejoins exons in pre-mRNAs.

#### RNA Transcription

The transcription process synthesizes an RNA copy of DNA. Proteins involved include multi-subunit RNA polymerases, transcription factors IIA, IIB, IID, IIE, IIF, IIH, and IIJ. Many transcription factors incorporate DNA-binding structural motifs which comprise either  $\alpha$ -helices or  $\beta$ -sheets that bind to the major groove of DNA. Four well-characterized structural motifs are helix-turn-helix, zinc finger, leucine zipper, and helix-loop-helix.

#### RNA Processing

Various proteins are necessary for processing of transcribed RNAs in the nucleus. Pre-mRNA processing steps include capping at the 5' end with methylguanosine, polyadenylating the 3' end, and splicing to remove introns. The spliceosomal complex is comprised of five small nuclear ribonucleoprotein particles (snRNPs) designated U1, U2, U4, U5, and U6. Each snRNP contains a single species of snRNA and about ten proteins. The RNA components of some snRNPs recognize and base-pair with intron consensus sequences. The protein components mediate spliceosome assembly and the splicing reaction. Autoantibodies to snRNP proteins are found in the blood of patients with systemic lupus erythematosus (Stryer, L. (1995) Biochemistry W.H. Freeman and Company, New York NY, p. 863).

Heterogeneous nuclear ribonucleoproteins (hnRNPs) have been identified that have roles in splicing, exporting of the mature RNAs to the cytoplasm, and mRNA translation (Biamonti, G. et al. (1998) Clin. Exp. Rheumatol. 16:317-326). Some examples of hnRNPs include the yeast proteins Hrp1p, involved in cleavage and polyadenylation at the 3' end of the RNA; Cbp80p, involved in capping the 5' end of the RNA; and Npl3p, a homolog of mammalian hnRNP A1, involved in export of mRNA from the nucleus (Shen, E.C. et al. (1998) Genes Dev. 12:679-691). hnRNPs have been shown to be important targets of the autoimmune response in rheumatic diseases (Biamonti, supra).

Many snRNP proteins, hnRNP proteins, and alternative splicing factors are characterized by an RNA recognition motif (RRM). (Reviewed in Birney, E. et al. (1993) Nucleic Acids Res. 21:5803-5816.) The RRM is about 80 amino acids in length and forms four  $\beta$ -strands and two  $\alpha$ -helices

arranged in an  $\alpha/\beta$  sandwich. The RRM contains a core RNP-1 octapeptide motif along with surrounding conserved sequences.

### RNA Stability and Degradation

RNA helicases alter and regulate RNA conformation and secondary structure by using energy derived from ATP hydrolysis to destabilize and unwind RNA duplexes. The most well-characterized and ubiquitous family of RNA helicases is the DEAD-box family, so named for the conserved B-type ATP-binding motif which is diagnostic of proteins in this family. Over 40 DEAD-box helicases have been identified in organisms as diverse as bacteria, insects, yeast, amphibians, mammals, and plants. DEAD-box helicases function in diverse processes such as translation initiation, splicing, ribosome assembly, and RNA editing, transport, and stability. Some DEAD-box helicases play tissue- and stage-specific roles in spermatogenesis and embryogenesis. (Reviewed in Linder, P. et al. (1989) Nature 337:121-122.)

Overexpression of the DEAD-box 1 protein (DDX1) may play a role in the progression of neuroblastoma (Nb) and retinoblastoma (Rb) tumors. Other DEAD-box helicases have been implicated either directly or indirectly in ultraviolet light-induced tumors, B cell lymphoma, and myeloid malignancies. (Reviewed in Godbout, R. et al. (1998) J. Biol. Chem. 273:21161-21168.)

Ribonucleases (RNases) catalyze the hydrolysis of phosphodiester bonds in RNA chains, thus cleaving the RNA. For example, RNase P is a ribonucleoprotein enzyme which cleaves the 5' end of pre-tRNAs as part of their maturation process. RNase H digests the RNA strand of an RNA/DNA hybrid. Such hybrids occur in cells invaded by retroviruses, and RNase H is an important enzyme in the retroviral replication cycle. RNase H domains are often found as a domain associated with reverse transcriptases. RNase activity in serum and cell extracts is elevated in a variety of cancers and infectious diseases (Schein, C.H. (1997) Nat. Biotechnol. 15:529-536). Regulation of RNase activity is being investigated as a means to control tumor angiogenesis, allergic reactions, viral infection and replication, and fungal infections.

### Protein Translation

The eukaryotic ribosome is composed of a 60S (large) subunit and a 40S (small) subunit, which together form the 80S ribosome. In addition to the 18S, 28S, 5S, and 5.8S rRNAs, the ribosome also contains more than fifty proteins. The ribosomal proteins have a prefix which denotes the subunit to which they belong, either L (large) or S (small). Three important sites are identified on the ribosome. The aminoacyl-tRNA site (A site) is where charged tRNAs (with the exception of the initiator-tRNA) bind on arrival at the ribosome. The peptidyl-tRNA site (P site) is where new peptide bonds are formed, as well as where the initiator tRNA binds. The exit site (E site) is where deacylated tRNAs bind prior to their release from the ribosome. (Translation is reviewed in Stryer, L. (1995)

Biochemistry, W.H. Freeman and Company, New York NY, pp. 875-908; and Lodish, H. et al. (1995)

Molecular Cell Biology, Scientific American Books, New York NY, pp. 119-138.)

### tRNA Charging

Protein biosynthesis depends on each amino acid forming a linkage with the appropriate tRNA. The aminoacyl-tRNA synthetases are responsible for the activation and correct attachment of an amino acid with its cognate tRNA. The 20 aminoacyl-tRNA synthetase enzymes can be divided into two structural classes, Class I and Class II. Autoantibodies against aminoacyl-tRNAs are generated by patients with dermatomyositis and polymyositis, and correlate strongly with complicating interstitial lung disease (ILD). These antibodies appear to be generated in response to viral infection, and coxsackie virus has been used to induce experimental viral myositis in animals.

### Translation Initiation

Initiation of translation can be divided into three stages. The first stage brings an initiator transfer RNA (Met-tRNA<sub>i</sub>) together with the 40S ribosomal subunit to form the 43S preinitiation complex. The second stage binds the 43S preinitiation complex to the mRNA, followed by migration of the complex to the correct AUG initiation codon. The third stage brings the 60S ribosomal subunit to the 40S subunit to generate an 80S ribosome at the initiation codon. Regulation of translation primarily involves the first and second stage in the initiation process (Pain, V.M. (1996) Eur. J. Biochem. 236:747-771).

Several initiation factors, many of which contain multiple subunits, are involved in bringing an initiator tRNA and 40S ribosomal subunit together. eIF2, a guanine nucleotide binding protein, recruits the initiator tRNA to the 40S ribosomal subunit. Only when eIF2 is bound to GTP does it associate with the initiator tRNA. eIF2B, a guanine nucleotide exchange protein, is responsible for converting eIF2 from the GDP-bound inactive form to the GTP-bound active form. Two other factors, eIF1A and eIF3 bind and stabilize the 40S subunit by interacting with 18S ribosomal RNA and specific ribosomal structural proteins. eIF3 is also involved in association of the 40S ribosomal subunit with mRNA. The Met-tRNA<sub>i</sub>, eIF1A, eIF3, and 40S ribosomal subunit together make up the 43S preinitiation complex (Pain, *supra*).

Additional factors are required for binding of the 43S preinitiation complex to an mRNA molecule, and the process is regulated at several levels. eIF4F is a complex consisting of three proteins: eIF4E, eIF4A, and eIF4G. eIF4E recognizes and binds to the mRNA 5'-terminal m<sup>7</sup>GTP cap, eIF4A is a bidirectional RNA-dependent helicase, and eIF4G is a scaffolding polypeptide. eIF4G has three binding domains. The N-terminal third of eIF4G interacts with eIF4E, the central third interacts with eIF4A, and the C-terminal third interacts with eIF3 bound to the 43S preinitiation complex. Thus, eIF4G acts as a bridge between the 40S ribosomal subunit and the mRNA (Hentze, M.W. (1997) Science 275:500-501).

The ability of eIF4F to initiate binding of the 43S preinitiation complex is regulated by

structural features of the mRNA. The mRNA molecule has an untranslated region (UTR) between the 5' cap and the AUG start codon. In some mRNAs this region forms secondary structures that impede binding of the 43S preinitiation complex. The helicase activity of eIF4A is thought to function in removing this secondary structure to facilitate binding of the 43S preinitiation complex (Pain, supra).

5     Translation Elongation

Elongation is the process whereby additional amino acids are joined to the initiator methionine to form the complete polypeptide chain. The elongation factors EF1 $\alpha$ , EF1 $\beta$   $\gamma$ , and EF2 are involved in elongating the polypeptide chain following initiation. EF1 $\alpha$  is a GTP-binding protein. In EF1 $\alpha$ 's GTP-bound form, it brings an aminoacyl-tRNA to the ribosome's A site. The amino acid attached to  
10   the newly arrived aminoacyl-tRNA forms a peptide bond with the initiator methionine. The GTP on EF1 $\alpha$  is hydrolyzed to GDP, and EF1 $\alpha$ -GDP dissociates from the ribosome. EF1 $\beta$   $\gamma$  binds EF1 $\alpha$ -GDP and induces the dissociation of GDP from EF1 $\alpha$ , allowing EF1 $\alpha$  to bind GTP and a new cycle to begin.

As subsequent aminoacyl-tRNAs are brought to the ribosome, EF-G, another GTP-binding protein, catalyzes the translocation of tRNAs from the A site to the P site and finally to the E site of the  
15   ribosome. This allows the processivity of translation.

Translation Termination

The release factor eRF carries out termination of translation. eRF recognizes stop codons in the mRNA, leading to the release of the polypeptide chain from the ribosome.

Post-Translational Pathways

20   Proteins may be modified after translation by the addition of phosphate, sugar, prenyl, fatty acid, and other chemical groups. These modifications are often required for proper protein activity. Enzymes involved in post-translational modification include kinases, phosphatases, glycosyltransferases, and prenyltransferases. The conformation of proteins may also be modified after translation by the introduction and rearrangement of disulfide bonds (rearrangement catalyzed by  
25   protein disulfide isomerase), the isomerization of proline sidechains by prolyl isomerase, and by interactions with molecular chaperone proteins.

Proteins may also be cleaved by proteases. Such cleavage may result in activation, inactivation, or complete degradation of the protein. Proteases include serine proteases, cysteine proteases, aspartic proteases, and metalloproteases. Signal peptidase in the endoplasmic reticulum  
30   (ER) lumen cleaves the signal peptide from membrane or secretory proteins that are imported into the ER. Ubiquitin proteases are associated with the ubiquitin conjugation system (UCS), a major pathway for the degradation of cellular proteins in eukaryotic cells and some bacteria. The UCS mediates the elimination of abnormal proteins and regulates the half-lives of important regulatory proteins that control cellular processes such as gene transcription and cell cycle progression. In the  
35   UCS pathway, proteins targeted for degradation are conjugated to a ubiquitin, a small heat stable



protein. Proteins involved in the UCS include ubiquitin-activating enzyme, ubiquitin-conjugating enzymes, ubiquitin-ligases, and ubiquitin C-terminal hydrolases. The ubiquitinated protein is then recognized and degraded by the proteasome, a large, multisubunit proteolytic enzyme complex, and ubiquitin is released for reutilization by ubiquitin protease.

#### 5 Lipid Metabolism

Lipids are water-insoluble, oily or greasy substances that are soluble in nonpolar solvents such as chloroform or ether. Neutral fats (triacylglycerols) serve as major fuels and energy stores. Polar lipids, such as phospholipids, sphingolipids, glycolipids, and cholesterol, are key structural components of cell membranes.

10 Lipid metabolism is involved in human diseases and disorders. In the arterial disease atherosclerosis, fatty lesions form on the inside of the arterial wall. These lesions promote the loss of arterial flexibility and the formation of blood clots (Guyton, A.C. Textbook of Medical Physiology (1991) W.B. Saunders Company, Philadelphia PA, pp.760-763). In Tay-Sachs disease, the GM<sub>2</sub> ganglioside (a sphingolipid) accumulates in lysosomes of the central nervous system due to a lack of the enzyme N-acetylhexosaminidase. Patients suffer nervous system degeneration leading to early death (Fauci, A.S. et al. (1998) Harrison's Principles of Internal Medicine McGraw-Hill, New York NY, p. 2171). The Niemann-Pick diseases are caused by defects in lipid metabolism. Niemann-Pick diseases types A and B are caused by accumulation of sphingomyelin (a sphingolipid) and other lipids in the central nervous system due to a defect in the enzyme sphingomyelinase, leading to neurodegeneration and lung disease. Niemann-Pick disease type C results from a defect in cholesterol transport, leading to the accumulation of sphingomyelin and cholesterol in lysosomes and a secondary reduction in sphingomyelinase activity. Neurological symptoms such as grand mal seizures, ataxia, and loss of previously learned speech, manifest 1-2 years after birth. A mutation in the NPC protein, which contains a putative cholesterol-sensing domain, was found in a mouse model of Niemann-Pick disease type C (Fauci, supra, p. 2175; Loftus, S.K. et al. (1997) *Science* 277:232-235). (Lipid metabolism is reviewed in Stryer, L. (1995) Biochemistry, W.H. Freeman and Company, New York NY; Lehninger, A. (1982) Principles of Biochemistry Worth Publishers, Inc., New York NY; and ExPASy "Biochemical Pathways" index of Boehringer Mannheim World Wide Web site.)

#### 25 Fatty Acid Synthesis

30 Fatty acids are long-chain organic acids with a single carboxyl group and a long non-polar hydrocarbon tail. Long-chain fatty acids are essential components of glycolipids, phospholipids, and cholesterol, which are building blocks for biological membranes, and of triglycerides, which are biological fuel molecules. Long-chain fatty acids are also substrates for eicosanoid production, and are important in the functional modification of certain complex carbohydrates and proteins. 16-carbon and 18-carbon fatty acids are the most common.

Fatty acid synthesis occurs in the cytoplasm. In the first step, acetyl-Coenzyme A (CoA) carboxylase (ACC) synthesizes malonyl-CoA from acetyl-CoA and bicarbonate. The enzymes which catalyze the remaining reactions are covalently linked into a single polypeptide chain, referred to as the multifunctional enzyme fatty acid synthase (FAS). FAS catalyzes the synthesis of palmitate from acetyl-CoA and malonyl-CoA. FAS contains acetyl transferase, malonyl transferase,  $\beta$ -ketoacetyl synthase, acyl carrier protein,  $\beta$ -ketoacyl reductase, dehydratase, enoyl reductase, and thioesterase activities. The final product of the FAS reaction is the 16-carbon fatty acid palmitate. Further elongation, as well as unsaturation, of palmitate by accessory enzymes of the ER produces the variety of long chain fatty acids required by the individual cell. These enzymes include a NADH-cytochrome  $b_5$  reductase, cytochrome  $b_5$ , and a desaturase.

#### Phospholipid and Triacylglycerol Synthesis

Triacylglycerols, also known as triglycerides and neutral fats, are major energy stores in animals. Triacylglycerols are esters of glycerol with three fatty acid chains. Glycerol-3-phosphate is produced from dihydroxyacetone phosphate by the enzyme glycerol phosphate dehydrogenase or from glycerol by glycerol kinase. Fatty acid-CoA's are produced from fatty acids by fatty acyl-CoA synthetases. Glycerol-3-phosphate is acylated with two fatty acyl-CoA's by the enzyme glycerol phosphate acyltransferase to give phosphatidate. Phosphatidate phosphatase converts phosphatidate to diacylglycerol, which is subsequently acylated to a triacylglycerol by the enzyme diglyceride acyltransferase. Phosphatidate phosphatase and diglyceride acyltransferase form a triacylglycerol synthetase complex bound to the ER membrane.

A major class of phospholipids are the phosphoglycerides, which are composed of a glycerol backbone, two fatty acid chains, and a phosphorylated alcohol. Phosphoglycerides are components of cell membranes. Principal phosphoglycerides are phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, and diphosphatidyl glycerol. Many enzymes involved in phosphoglyceride synthesis are associated with membranes (Meyers, R.A. (1995) Molecular Biology and Biotechnology, VCH Publishers Inc., New York NY, pp. 494-501). Phosphatidate is converted to CDP-diacylglycerol by the enzyme phosphatidate cytidylyltransferase (ExPASy ENZYME EC 2.7.7.41). Transfer of the diacylglycerol group from CDP-diacylglycerol to serine to yield phosphatidyl serine, or to inositol to yield phosphatidyl inositol, is catalyzed by the enzymes CDP-diacylglycerol-serine O-phosphatidyltransferase and CDP-diacylglycerol-inositol 3-phosphatidyltransferase, respectively (ExPASy ENZYME EC 2.7.8.8; ExPASy ENZYME EC 2.7.8.11). The enzyme phosphatidyl serine decarboxylase catalyzes the conversion of phosphatidyl serine to phosphatidyl ethanolamine, using a pyruvate cofactor (Voelker, D.R. (1997) *Biochim. Biophys. Acta* 1348:236-244). Phosphatidyl choline is formed using diet-derived choline by the reaction of CDP-choline with 1,2-diacylglycerol, catalyzed by diacylglycerol cholinephosphotransferase (ExPASy ENZYME 2.7.8.2).

### Sterol, Steroid, and Isoprenoid Metabolism

Cholesterol, composed of four fused hydrocarbon rings with an alcohol at one end, moderates the fluidity of membranes in which it is incorporated. In addition, cholesterol is used in the synthesis of steroid hormones such as cortisol, progesterone, estrogen, and testosterone. Bile salts derived from cholesterol facilitate the digestion of lipids. Cholesterol in the skin forms a barrier that prevents excess water evaporation from the body. Farnesyl and geranylgeranyl groups, which are derived from cholesterol biosynthesis intermediates, are post-translationally added to signal transduction proteins such as ras and protein-targeting proteins such as rab. These modifications are important for the activities of these proteins (Guyton, *supra*; Stryer, *supra*, pp. 279-280, 691-702, 934).

Mammals obtain cholesterol derived from both *de novo* biosynthesis and the diet. The liver is the major site of cholesterol biosynthesis in mammals. Two acetyl-CoA molecules initially condense to form acetoacetyl-CoA, catalyzed by a thiolase. Acetoacetyl-CoA condenses with a third acetyl-CoA to form hydroxymethylglutaryl-CoA (HMG-CoA), catalyzed by HMG-CoA synthase. Conversion of HMG-CoA to cholesterol is accomplished via a series of enzymatic steps known as the mevalonate pathway. The rate-limiting step is the conversion of HMG-CoA to mevalonate by HMG-CoA reductase. The drug lovastatin, a potent inhibitor of HMG-CoA reductase, is given to patients to reduce their serum cholesterol levels. Other mevalonate pathway enzymes include mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, isopentenylidiphosphate isomerase, dimethylallyl transferase, geranyl transferase, farnesyl-diphosphate farnesyltransferase, squalene monooxygenase, lanosterol synthase, lanosterol oxidase, and 7-dehydrocholesterol reductase.

Cholesterol is used in the synthesis of steroid hormones such as cortisol, progesterone, aldosterone, estrogen, and testosterone. First, cholesterol is converted to pregnenolone by cholesterol monooxygenases. The other steroid hormones are synthesized from pregnenolone by a series of enzyme-catalyzed reactions including oxidations, isomerizations, hydroxylations, reductions, and demethylations. Examples of these enzymes include steroid  $\Delta$ -isomerase,  $3\beta$ -hydroxy- $\Delta^5$ -steroid dehydrogenase, steroid 21-monooxygenase, steroid 19-hydroxylase, and  $3\beta$ -hydroxysteroid dehydrogenase. Cholesterol is also the precursor to vitamin D.

Numerous compounds contain 5-carbon isoprene units derived from the mevalonate pathway intermediate isopentenyl pyrophosphate. Isoprenoid groups are found in vitamin K, ubiquinone, retinal, dolichol phosphate (a carrier of oligosaccharides needed for N-linked glycosylation), and farnesyl and geranylgeranyl groups that modify proteins. Enzymes involved include farnesyl transferase, polyprenyl transferases, dolichyl phosphatase, and dolichyl kinase.

### Sphingolipid Metabolism

Sphingolipids are an important class of membrane lipids that contain sphingosine, a long chain amino alcohol. They are composed of one long-chain fatty acid, one polar head alcohol, and

sphingosine or sphingosine derivative. The three classes of sphingolipids are sphingomyelins, cerebrosidases, and gangliosides. Sphingomyelins, which contain phosphocholine or phosphoethanolamine as their head group, are abundant in the myelin sheath surrounding nerve cells. Galactocerebrosides, which contain a glucose or galactose head group, are characteristic of the brain.

- 5 Other cerebrosidases are found in nonneural tissues. Gangliosides, whose head groups contain multiple sugar units, are abundant in the brain, but are also found in nonneural tissues.

Sphingolipids are built on a sphingosine backbone. Sphingosine is acylated to ceramide by the enzyme sphingosine acetyltransferase. Ceramide and phosphatidyl choline are converted to sphingomyelin by the enzyme ceramide choline phosphotransferase. Cerebrosides are synthesized by the linkage of glucose or galactose to ceramide by a transferase. Sequential addition of sugar residues to ceramide by transferase enzymes yields gangliosides.

#### Eicosanoid Metabolism

- Eicosanoids, including prostaglandins, prostacyclin, thromboxanes, and leukotrienes, are 20-carbon molecules derived from fatty acids. Eicosanoids are signaling molecules which have roles in pain, fever, and inflammation. The precursor of all eicosanoids is arachidonate, which is generated from phospholipids by phospholipase  $A_2$  and from diacylglycerols by diacylglycerol lipase. Leukotrienes are produced from arachidonate by the action of lipoxygenases. Prostaglandin synthase, reductases, and isomerases are responsible for the synthesis of the prostaglandins. Prostaglandins have roles in inflammation, blood flow, ion transport, synaptic transmission, and sleep. Prostacyclin and the thromboxanes are derived from a precursor prostaglandin by the action of prostacyclin synthase and thromboxane synthases, respectively.

#### Ketone Body Metabolism

- Pairs of acetyl-CoA molecules derived from fatty acid oxidation in the liver can condense to form acetoacetyl-CoA, which subsequently forms acetoacetate, D-3-hydroxybutyrate, and acetone. These three products are known as ketone bodies. Enzymes involved in ketone body metabolism include HMG-CoA synthetase, HMG-CoA cleavage enzyme, D-3-hydroxybutyrate dehydrogenase, acetoacetate decarboxylase, and 3-ketoacyl-CoA transferase. Ketone bodies are a normal fuel supply of the heart and renal cortex. Acetoacetate produced by the liver is transported to cells where the acetoacetate is converted back to acetyl-CoA and enters the citric acid cycle. In times of starvation, ketone bodies produced from stored triacylglycerols become an important fuel source, especially for the brain. Abnormally high levels of ketone bodies are observed in diabetics. Diabetic coma can result if ketone body levels become too great.

#### Lipid Mobilization

- Within cells, fatty acids are transported by cytoplasmic fatty acid binding proteins (Online Mendelian Inheritance in Man (OMIM) \*134650 Fatty Acid-Binding Protein 1, Liver; FABP1).

Diazepam binding inhibitor (DBI), also known as endozepine and acyl CoA-binding protein, is an endogenous  $\gamma$ -aminobutyric acid (GABA) receptor ligand which is thought to down-regulate the effects of GABA. DBI binds medium- and long-chain acyl-CoA esters with very high affinity and may function as an intracellular carrier of acyl-CoA esters (OMIM \*125950 Diazepam Binding Inhibitor;

5 DBI; PROSITE PDOC00686 Acyl-CoA-binding protein signature).

Fat stored in liver and adipose triglycerides may be released by hydrolysis and transported in the blood. Free fatty acids are transported in the blood by albumin. Triacylglycerols and cholesterol esters in the blood are transported in lipoprotein particles. The particles consist of a core of hydrophobic lipids surrounded by a shell of polar lipids and apolipoproteins. The protein components serve in the solubilization of hydrophobic lipids and also contain cell-targeting signals. Lipoproteins include chylomicrons, chylomicron remnants, very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). There is a strong inverse correlation between the levels of plasma HDL and risk of premature coronary heart disease.

15 Triacylglycerols in chylomicrons and VLDL are hydrolyzed by lipoprotein lipases that line blood vessels in muscle and other tissues that use fatty acids. Cell surface LDL receptors bind LDL particles which are then internalized by endocytosis. Absence of the LDL receptor, the cause of the disease familial hypercholesterolemia, leads to increased plasma cholesterol levels and ultimately to atherosclerosis. Plasma cholesteryl ester transfer protein mediates the transfer of cholesteryl esters from HDL to apolipoprotein B-containing lipoproteins. Cholesteryl ester transfer protein is important in the reverse cholesterol transport system and may play a role in atherosclerosis (Yamashita, S. et al. (1997) *Curr. Opin. Lipidol.* 8:101-110). Macrophage scavenger receptors, which bind and internalize modified lipoproteins, play a role in lipid transport and may contribute to atherosclerosis (Greaves, D.R. et al. (1998) *Curr. Opin. Lipidol.* 9:425-432).

25 Proteins involved in cholesterol uptake and biosynthesis are tightly regulated in response to cellular cholesterol levels. The sterol regulatory element binding protein (SREBP) is a sterol-responsive transcription factor. Under normal cholesterol conditions, SREBP resides in the ER membrane. When cholesterol levels are low, a regulated cleavage of SREBP occurs which releases the extracellular domain of the protein. This cleaved domain is then transported to the nucleus where it activates the transcription of the LDL receptor gene, and genes encoding enzymes of cholesterol synthesis, by binding the sterol regulatory element (SRE) upstream of the genes (Yang, J. et al. (1995) *J. Biol. Chem.* 270:12152-12161). Regulation of cholesterol uptake and biosynthesis also occurs via the oxysterol-binding protein (OSBP). OSBP is a high-affinity intracellular receptor for a variety of oxysterols that down-regulate cholesterol synthesis and stimulate cholesterol esterification (Lagace, T.A. et al. (1997) *Biochem. J.* 326:205-213).

### Beta-oxidation

Mitochondrial and peroxisomal beta-oxidation enzymes degrade saturated and unsaturated fatty acids by sequential removal of two-carbon units from CoA-activated fatty acids. The main beta-oxidation pathway degrades both saturated and unsaturated fatty acids while the auxiliary pathway performs additional steps required for the degradation of unsaturated fatty acids.

The pathways of mitochondrial and peroxisomal beta-oxidation use similar enzymes, but have different substrate specificities and functions. Mitochondria oxidize short-, medium-, and long-chain fatty acids to produce energy for cells. Mitochondrial beta-oxidation is a major energy source for cardiac and skeletal muscle. In liver, it provides ketone bodies to the peripheral circulation when glucose levels are low as in starvation, endurance exercise, and diabetes (Eaton, S. et al. (1996) Biochem. J. 320:345-357). Peroxisomes oxidize medium-, long-, and very-long-chain fatty acids, dicarboxylic fatty acids, branched fatty acids, prostaglandins, xenobiotics, and bile acid intermediates. The chief roles of peroxisomal beta-oxidation are to shorten toxic lipophilic carboxylic acids to facilitate their excretion and to shorten very-long-chain fatty acids prior to mitochondrial beta-oxidation (Mannaerts, G.P. and P.P. van Veldhoven (1993) Biochimic 75:147-158).

Enzymes involved in beta-oxidation include acyl CoA synthetase, carnitine acyltransferase, acyl CoA dehydrogenases, enoyl CoA hydratases, L-3-hydroxyacyl CoA dehydrogenase,  $\beta$ -ketothiolase, 2,4-dienoyl CoA reductase, and isomerase.

### Lipid Cleavage and Degradation

Triglycerides are hydrolyzed to fatty acids and glycerol by lipases. Lysophospholipases (LPLs) are widely distributed enzymes that metabolize intracellular lipids, and occur in numerous isoforms. Small isoforms, approximately 15-30 kD, function as hydrolases; large isoforms, those exceeding 60 kD, function both as hydrolases and transacylases. A particular substrate for LPLs, lysophosphatidylcholine, causes lysis of cell membranes when it is formed or imported into a cell. LPLs are regulated by lipid factors including acylcarnitine, arachidonic acid, and phosphatidic acid. These lipid factors are signaling molecules important in numerous pathways, including the inflammatory response. (Anderson, R. et al. (1994) Toxicol. Appl. Pharmacol. 125:176-183; Selle, H. et al. (1993); Eur. J. Biochem. 212:411-416.)

The secretory phospholipase A<sub>2</sub> (PLA<sub>2</sub>) superfamily comprises a number of heterogeneous enzymes whose common feature is to hydrolyze the sn-2 fatty acid acyl ester bond of phosphoglycerides. Hydrolysis of the glycerophospholipids releases free fatty acids and lysophospholipids. PLA<sub>2</sub> activity generates precursors for the biosynthesis of biologically active lipids, hydroxy fatty acids, and platelet-activating factor. PLA<sub>2</sub> hydrolysis of the sn-2 ester bond in phospholipids generates free fatty acids, such as arachidonic acid and lysophospholipids.

### Carbon and Carbohydrate Metabolism

Carbohydrates, including sugars or saccharides, starch, and cellulose, are aldehyde or ketone compounds with multiple hydroxyl groups. The importance of carbohydrate metabolism is demonstrated by the sensitive regulatory system in place for maintenance of blood glucose levels. Two pancreatic hormones, insulin and glucagon, promote increased glucose uptake and storage by cells, and increased glucose release from cells, respectively. Carbohydrates have three important roles in mammalian cells. First, carbohydrates are used as energy stores, fuels, and metabolic intermediates. Carbohydrates are broken down to form energy in glycolysis and are stored as glycogen for later use. Second, the sugars deoxyribose and ribose form part of the structural support of DNA and RNA, respectively. Third, carbohydrate modifications are added to secreted and membrane proteins and lipids as they traverse the secretory pathway. Cell surface carbohydrate-containing macromolecules, including glycoproteins, glycolipids, and transmembrane proteoglycans, mediate adhesion with other cells and with components of the extracellular matrix. The extracellular matrix is comprised of diverse glycoproteins, glycosaminoglycans (GAGs), and carbohydrate-binding proteins which are secreted from the cell and assembled into an organized meshwork in close association with the cell surface. The interaction of the cell with the surrounding matrix profoundly influences cell shape, strength, flexibility, motility, and adhesion. These dynamic properties are intimately associated with signal transduction pathways controlling cell proliferation and differentiation, tissue construction, and embryonic development.

Carbohydrate metabolism is altered in several disorders including diabetes mellitus, hyperglycemia, hypoglycemia, galactosemia, galactokinase deficiency, and UDP-galactose-4-epimerase deficiency (Fauci, A.S. et al. (1998) Harrison's Principles of Internal Medicine, McGraw-Hill, New York NY, pp. 2208-2209). Altered carbohydrate metabolism is associated with cancer. Reduced GAG and proteoglycan expression is associated with human lung carcinomas (Nackaerts, K. et al. (1997) *Int. J. Cancer* 74:335-345). The carbohydrate determinants sialyl Lewis A and sialyl Lewis X are frequently expressed on human cancer cells (Kannagi, R. (1997) *Glycoconj. J.* 14:577-584). Alterations of the N-linked carbohydrate core structure of cell surface glycoproteins are linked to colon and pancreatic cancers (Schwarz, R.E. et al. (1996) *Cancer Lett.* 107:285-291). Reduced expression of the Sda blood group carbohydrate structure in cell surface glycolipids and glycoproteins is observed in gastrointestinal cancer (Dohi, T. et al. (1996) *Int. J. Cancer* 67:626-663). (Carbon and carbohydrate metabolism is reviewed in Stryer, L. (1995) Biochemistry W.H. Freeman and Company, New York NY; Lehninger, A.L. (1982) Principles of Biochemistry Worth Publishers Inc., New York NY; and Lodish, H. et al. (1995) Molecular Cell Biology Scientific American Books, New York NY.) Glycolysis

Enzymes of the glycolytic pathway convert the sugar glucose to pyruvate while simultaneously producing ATP. The pathway also provides building blocks for the synthesis of cellular components

such as long-chain fatty acids. After glycolysis, pyruvate is converted to acetyl-Coenzyme A, which, in aerobic organisms, enters the citric acid cycle. Glycolytic enzymes include hexokinase, phosphoglucose isomerase, phosphofructokinase, aldolase, triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglyceromutase, enolase, and pyruvate kinase. Of these, phosphofructokinase, hexokinase, and pyruvate kinase are important in regulating the rate of glycolysis.

#### Gluconeogenesis

Gluconeogenesis is the synthesis of glucose from noncarbohydrate precursors such as lactate and amino acids. The pathway, which functions mainly in times of starvation and intense exercise, occurs mostly in the liver and kidney. Responsible enzymes include pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose 1,6-bisphosphatase, and glucose-6-phosphatase.

#### Pentose Phosphate Pathway

Pentose phosphate pathway enzymes are responsible for generating the reducing agent NADPH, while at the same time oxidizing glucose-6-phosphate to ribose-5-phosphate. Ribose-5-phosphate and its derivatives become part of important biological molecules such as ATP, Coenzyme A, NAD<sup>+</sup>, FAD, RNA, and DNA. The pentose phosphate pathway has both oxidative and non-oxidative branches. The oxidative branch steps, which are catalyzed by the enzymes glucose-6-phosphate dehydrogenase, lactonase, and 6-phosphogluconate dehydrogenase, convert glucose-6-phosphate and NADP<sup>+</sup> to ribulose-6-phosphate and NADPH. The non-oxidative branch steps, which are catalyzed by the enzymes phosphopentose isomerase, phosphopentose epimerase, transketolase, and transaldolase, allow the interconversion of three-, four-, five-, six-, and seven-carbon sugars.

#### Glucuronate Metabolism

Glucuronate is a monosaccharide which, in the form of D-glucuronic acid, is found in the GAGs chondroitin and dermatan. D-glucuronic acid is also important in the detoxification and excretion of foreign organic compounds such as phenol. Enzymes involved in glucuronate metabolism include UDP-glucose dehydrogenase and glucuronate reductase.

#### Disaccharide Metabolism

Disaccharides must be hydrolyzed to monosaccharides to be digested. Lactose, a disaccharide found in milk, is hydrolyzed to galactose and glucose by the enzyme lactase. Maltose is derived from plant starch and is hydrolyzed to glucose by the enzyme maltase. Sucrose is derived from plants and is hydrolyzed to glucose and fructose by the enzyme sucrase. Trehalose, a disaccharide found mainly in insects and mushrooms, is hydrolyzed to glucose by the enzyme trehalase (OMIM \*275360 Trehalase; Ruf, J. et al. (1990) J. Biol. Chem. 265:15034-15039). Lactase, maltase, sucrase, and trehalase are bound to mucosal cells lining the small intestine, where they participate in the digestion of dietary disaccharides. The enzyme lactose synthetase, composed of the catalytic subunit galactosyltransferase



and the modifier subunit  $\alpha$ -lactalbumin, converts UDP-galactose and glucose to lactose in the mammary glands.

#### Glycogen, Starch, and Chitin Metabolism

Glycogen is the storage form of carbohydrates in mammals. Mobilization of glycogen maintains glucose levels between meals and during muscular activity. Glycogen is stored mainly in the liver and in skeletal muscle in the form of cytoplasmic granules. These granules contain enzymes that catalyze the synthesis and degradation of glycogen, as well as enzymes that regulate these processes. Enzymes that catalyze the degradation of glycogen include glycogen phosphorylase, a transferase,  $\alpha$ -1,6-glucosidase, and phosphoglucomutase. Enzymes that catalyze the synthesis of glycogen include UDP-glucose pyrophosphorylase, glycogen synthetase, a branching enzyme, and nucleoside diphosphokinase. The enzymes of glycogen synthesis and degradation are tightly regulated by the hormones insulin, glucagon, and epinephrine. Starch, a plant-derived polysaccharide, is hydrolyzed to maltose, maltotriose, and  $\alpha$ -dextrin by  $\alpha$ -amylase, an enzyme secreted by the salivary glands and pancreas. Chitin is a polysaccharide found in insects and crustacea. A chitotriosidase is secreted by macrophages and may play a role in the degradation of chitin-containing pathogens (Boot, R.G. et al. (1995) J. Biol. Chem. 270:26252-26256).

#### Peptidoglycans and Glycosaminoglycans

Glycosaminoglycans (GAGs) are anionic linear unbranched polysaccharides composed of repetitive disaccharide units. These repetitive units contain a derivative of an amino sugar, either glucosamine or galactosamine. GAGs exist free or as part of proteoglycans, large molecules composed of a core protein attached to one or more GAGs. GAGs are found on the cell surface, inside cells, and in the extracellular matrix. Changes in GAG levels are associated with several autoimmune diseases including autoimmune thyroid disease, autoimmune diabetes mellitus, and systemic lupus erythematosus (Hansen, C. et al. (1996) Clin. Exp. Rheum. 14 (Suppl. 15):S59-S67). GAGs include chondroitin sulfate, keratan sulfate, heparin, heparan sulfate, dermatan sulfate, and hyaluronan.

The GAG hyaluronan (HA) is found in the extracellular matrix of many cells, especially in soft connective tissues, and is abundant in synovial fluid (Pitsillides, A.A. et al. (1993) Int. J. Exp. Pathol. 74:27-34). HA seems to play important roles in cell regulation, development, and differentiation (Laurent, T.C. and J.R. Fraser (1992) FASEB J. 6:2397-2404). Hyaluronidase is an enzyme that degrades HA to oligosaccharides. Hyaluronidases may function in cell adhesion, infection, angiogenesis, signal transduction, reproduction, cancer, and inflammation.

Proteoglycans, also known as peptidoglycans, are found in the extracellular matrix of connective tissues such as cartilage and are essential for distributing the load in weight-bearing joints. Cell-surface-attached proteoglycans anchor cells to the extracellular matrix. Both extracellular and cell-surface proteoglycans bind growth factors, facilitating their binding to cell-surface receptors and

subsequent triggering of signal transduction pathways.

### Amino Acid and Nitrogen Metabolism

$\text{NH}_4^+$  is assimilated into amino acids by the actions of two enzymes, glutamate dehydrogenase and glutamine synthetase. The carbon skeletons of amino acids come from the intermediates of glycolysis, the pentose phosphate pathway, or the citric acid cycle. Of the twenty amino acids used in proteins, humans can synthesize only thirteen (nonessential amino acids). The remaining nine must come from the diet (essential amino acids). Enzymes involved in nonessential amino acid biosynthesis include glutamate kinase dehydrogenase, pyrroline carboxylate reductase, asparagine synthetase, phenylalanine oxygenase, methionine adenosyltransferase, adenosylhomocysteinase, cystathionine  $\beta$ -synthase, cystathionine  $\gamma$ -lyase, phosphoglycerate dehydrogenase, phosphoserine transaminase, phosphoserine phosphatase, serine hydroxymethyltransferase, and glycine synthase.

Metabolism of amino acids takes place almost entirely in the liver, where the amino group is removed by aminotransferases (transaminases), for example, alanine aminotransferase. The amino group is transferred to  $\alpha$ -ketoglutarate to form glutamate. Glutamate dehydrogenase converts glutamate to  $\text{NH}_4^+$  and  $\alpha$ -ketoglutarate.  $\text{NH}_4^+$  is converted to urea by the urea cycle which is catalyzed by the enzymes arginase, ornithine transcarbamoylase, arginosuccinate synthetase, and arginosuccinase. Carbamoyl phosphate synthetase is also involved in urea formation. Enzymes involved in the metabolism of the carbon skeleton of amino acids include serine dehydratase, asparaginase, glutaminase, propionyl CoA carboxylase, methylmalonyl CoA mutase, branched-chain  $\alpha$ -keto dehydrogenase complex, isovaleryl CoA dehydrogenase,  $\beta$ -methylcrotonyl CoA carboxylase, phenylalanine hydroxylase, p-hydroxyphenylpyruvate hydroxylase, and homogentisate oxidase.

Polyamines, which include spermidine, putrescine, and spermine, bind tightly to nucleic acids and are abundant in rapidly proliferating cells. Enzymes involved in polyamine synthesis include ornithine decarboxylase.

Diseases involved in amino acid and nitrogen metabolism include hyperammonemia, carbamoyl phosphate synthetase deficiency, urea cycle enzyme deficiencies, methylmalonic aciduria, maple syrup disease, alcaptonuria, and phenylketonuria.

### Energy Metabolism

Cells derive energy from metabolism of ingested compounds that may be roughly categorized as carbohydrates, fats, or proteins. Energy is also stored in polymers such as triglycerides (fats) and glycogen (carbohydrates). Metabolism proceeds along separate reaction pathways connected by key intermediates such as acetyl coenzyme A (acetyl-CoA). Metabolic pathways feature anaerobic and aerobic degradation, coupled with the energy-requiring reactions such as phosphorylation of adenosine diphosphate (ADP) to the triphosphate (ATP) or analogous phosphorylations of guanosine

(GDP/GTP), uridine (UDP/UTP), or cytidine (CDP/CTP). Subsequent dephosphorylation of the triphosphate drives reactions needed for cell maintenance, growth, and proliferation.

Digestive enzymes convert carbohydrates and sugars to glucose; fructose and galactose are converted in the liver to glucose. Enzymes involved in these conversions include galactose-1-phosphate uridyl transferase and UDP-galactose-4 epimerase. In the cytoplasm, glycolysis converts glucose to pyruvate in a series of reactions coupled to ATP synthesis.

Pyruvate is transported into the mitochondria and converted to acetyl-CoA for oxidation via the citric acid cycle, involving pyruvate dehydrogenase components, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase. Enzymes involved in the citric acid cycle include: citrate synthetase, aconitases, isocitrate dehydrogenase, alpha-ketoglutarate dehydrogenase complex including transsuccinylases, succinyl CoA synthetase, succinate dehydrogenase, fumarases, and malate dehydrogenase. Acetyl CoA is oxidized to CO<sub>2</sub> with concomitant formation of NADH, FADH<sub>2</sub>, and GTP. In oxidative phosphorylation, the transport of electrons from NADH and FADH<sub>2</sub> to oxygen by dehydrogenases is coupled to the synthesis of ATP from ADP and P<sub>i</sub> by the F<sub>0</sub>F<sub>1</sub> ATPase complex in the mitochondrial inner membrane. Enzyme complexes responsible for electron transport and ATP synthesis include the F<sub>0</sub>F<sub>1</sub> ATPase complex, ubiquinone(CoQ)-cytochrome c reductase, ubiquinone reductase, cytochrome b, cytochrome c<sub>1</sub>, FeS protein, and cytochrome c oxidase.

Triglycerides are hydrolyzed to fatty acids and glycerol by lipases. Glycerol is then phosphorylated to glycerol-3-phosphate by glycerol kinase and glycerol phosphate dehydrogenase, and degraded by the glycolysis. Fatty acids are transported into the mitochondria as fatty acyl-carnitine esters and undergo oxidative degradation.

In addition to metabolic disorders such as diabetes and obesity, disorders of energy metabolism are associated with cancers (Dorward, A. et al. (1997) *J. Bioenerg. Biomembr.* 29:385-392), autism (Lombard, J. (1998) *Med. Hypotheses* 50:497-500), neurodegenerative disorders (Alexi, T. et al. (1998) *Neuroreport* 9:R57-64), and neuromuscular disorders (DiMauro, S. et al. (1998) *Biochim. Biophys. Acta* 1366:199-210). The myocardium is heavily dependent on oxidative metabolism, so metabolic dysfunction often leads to heart disease (DiMauro, S. and M. Hirano (1998) *Curr. Opin. Cardiol.* 13:190-197).

For a review of energy metabolism enzymes and intermediates, see Stryer, L. et al. (1995) *Biochemistry*, W.H. Freeman and Co., San Francisco CA, pp. 443-652. For a review of energy metabolism regulation, see Lodish, H. et al. (1995) *Molecular Cell Biology*, Scientific American Books, New York NY, pp. 744-770.

#### Cofactor Metabolism

Cofactors, including coenzymes and prosthetic groups, are small molecular weight inorganic or organic compounds that are required for the action of an enzyme. Many cofactors contain vitamins

as a component. Cofactors include thiamine pyrophosphate, flavin adenine dinucleotide, flavin mononucleotide, nicotinamide adenine dinucleotide, pyridoxal phosphate, coenzyme A, tetrahydrofolate, lipamide, and heme. The vitamins biotin and cobalamin are associated with enzymes as well. Heme, a prosthetic group found in myoglobin and hemoglobin, consists of

5 protoporphyrin group bound to iron. Porphyrin groups contain four substituted pyrroles covalently joined in a ring, often with a bound metal atom. Enzymes involved in porphyrin synthesis include  $\delta$ -aminolevulinic synthase,  $\delta$ -aminolevulinic dehydratase, uroporphyrinogen decarboxylase, and uroporphyrinogen decarboxylase. Deficiencies in heme formation cause porphyrias. Heme is broken down as a part of erythrocyte turnover. Enzymes involved in heme degradation include heme oxygenase and biliverdin reductase.

10 Iron is a required cofactor for many enzymes. Besides the heme-containing enzymes, iron is found in iron-sulfur clusters in proteins including aconitase, succinate dehydrogenase, and NADH-Q reductase. Iron is transported in the blood by the protein transferrin. Binding of transferrin to the transferrin receptor on cell surfaces allows uptake by receptor mediated endocytosis. Cytosolic iron is bound to ferritin protein.

15 A molybdenum-containing cofactor (molybdopterin) is found in enzymes including sulfite oxidase, xanthine dehydrogenase, and aldehyde oxidase. Molybdopterin biosynthesis is performed by two molybdenum cofactor synthesizing enzymes. Deficiencies in these enzymes cause mental retardation and lens dislocation. Other diseases caused by defects in cofactor metabolism include pernicious anemia and methylmalonic aciduria.

## 20 Secretion and Trafficking

Eukaryotic cells are bound by a lipid bilayer membrane and subdivided into functionally distinct, membrane bound compartments. The membranes maintain the essential differences between the cytosol, the extracellular environment, and the luminal space of each intracellular organelle. As lipid membranes are highly impermeable to most polar molecules, transport of essential nutrients,

25 metabolic waste products, cell signaling molecules, macromolecules and proteins across lipid membranes and between organelles must be mediated by a variety of transport-associated molecules.

## Protein Trafficking

In eukaryotes, some proteins are synthesized on ER-bound ribosomes, co-translationally imported into the ER, delivered from the ER to the Golgi complex for post-translational processing and

30 sorting, and transported from the Golgi to specific intracellular and extracellular destinations. All cells possess a constitutive transport process which maintains homeostasis between the cell and its environment. In many differentiated cell types, the basic machinery is modified to carry out specific transport functions. For example, in endocrine glands, hormones and other secreted proteins are packaged into secretory granules for regulated exocytosis to the cell exterior. In macrophage, foreign

35 extracellular material is engulfed (phagocytosis) and delivered to lysosomes for degradation. In fat and

muscle cells, glucose transporters are stored in vesicles which fuse with the plasma membrane only in response to insulin stimulation.

### The Secretory Pathway

Synthesis of most integral membrane proteins, secreted proteins, and proteins destined for the lumen of a particular organelle occurs on ER-bound ribosomes. These proteins are co-translationally imported into the ER. The proteins leave the ER via membrane-bound vesicles which bud off the ER at specific sites and fuse with each other (homotypic fusion) to form the ER-Golgi Intermediate Compartment (ERGIC). The ERGIC matures progressively through the *cis*, *medial*, and *trans* cisternal stacks of the Golgi, modifying the enzyme composition by retrograde transport of specific Golgi enzymes. In this way, proteins moving through the Golgi undergo post-translational modification, such as glycosylation. The final Golgi compartment is the Trans-Golgi Network (TGN), where both membrane and luminal proteins are sorted for their final destination. Transport vesicles destined for intracellular compartments, such as the lysosome, bud off the TGN. What remains is a secretory vesicle which contains proteins destined for the plasma membrane, such as receptors, adhesion molecules, and ion channels, and secretory proteins, such as hormones, neurotransmitters, and digestive enzymes. Secretory vesicles eventually fuse with the plasma membrane (Glick, B.S. and V. Malhotra (1998) Cell 95:883-889).

The secretory process can be constitutive or regulated. Most cells have a constitutive pathway for secretion, whereby vesicles derived from maturation of the TGN require no specific signal to fuse with the plasma membrane. In many cells, such as endocrine cells, digestive cells, and neurons, vesicle pools derived from the TGN collect in the cytoplasm and do not fuse with the plasma membrane until they are directed to by a specific signal.

### Endocytosis

Endocytosis, wherein cells internalize material from the extracellular environment, is essential for transmission of neuronal, metabolic, and proliferative signals; uptake of many essential nutrients; and defense against invading organisms. Most cells exhibit two forms of endocytosis. The first, phagocytosis, is an actin-driven process exemplified in macrophage and neutrophils. Material to be endocytosed contacts numerous cell surface receptors which stimulate the plasma membrane to extend and surround the particle, enclosing it in a membrane-bound phagosome. In the mammalian immune system, IgG-coated particles bind Fc receptors on the surface of phagocytic leukocytes. Activation of the Fc receptors initiates a signal cascade involving src-family cytosolic kinases and the monomeric GTP-binding (G) protein Rho. The resulting actin reorganization leads to phagocytosis of the particle. This process is an important component of the humoral immune response, allowing the processing and presentation of bacterial-derived peptides to antigen-specific T-lymphocytes.

The second form of endocytosis, pinocytosis, is a more generalized uptake of material from the

external milieu. Like phagocytosis, pinocytosis is activated by ligand binding to cell surface receptors. Activation of individual receptors stimulates an internal response that includes coalescence of the receptor-ligand complexes and formation of clathrin-coated pits. Invagination of the plasma membrane at clathrin-coated pits produces an endocytic vesicle within the cell cytoplasm. These vesicles undergo

5 homotypic fusion to form an early endosomal (EE) compartment. The tubulovesicular EE serves as a sorting site for incoming material. ATP-driven proton pumps in the EE membrane lowers the pH of the EE lumen (pH 6.3-6.8). The acidic environment causes many ligands to dissociate from their receptors. The receptors, along with membrane and other integral membrane proteins, are recycled back to the plasma membrane by budding off the tubular extensions of the EE in recycling vesicles (RV). This

10 selective removal of recycled components produces a carrier vesicle containing ligand and other material from the external environment. The carrier vesicle fuses with TGN-derived vesicles which contain hydrolytic enzymes. The acidic environment of the resulting late endosome (LE) activates the hydrolytic enzymes which degrade the ligands and other material. As digestion takes place, the LE fuses with the lysosome where digestion is completed (Mellman, I. (1996) Annu. Rev. Cell Dev. Biol.

15 12:575-625).

Recycling vesicles may return directly to the plasma membrane. Receptors internalized and returned directly to the plasma membrane have a turnover rate of 2-3 minutes. Some RVs undergo microtubule-directed relocation to a perinuclear site, from which they then return to the plasma membrane. Receptors following this route have a turnover rate of 5-10 minutes. Still other RVs are

20 retained within the cell until an appropriate signal is received (Mellman, supra; and James, D.E. et al. (1994) Trends Cell Biol. 4:120-126).

#### Vesicle Formation

Several steps in the transit of material along the secretory and endocytic pathways require the formation of transport vesicles. Specifically, vesicles form at the transitional endoplasmic reticulum

25 (tER), the rim of Golgi cisternae, the face of the Trans-Golgi Network (TGN), the plasma membrane (PM), and tubular extensions of the endosomes. The process begins with the budding of a vesicle out of the donor membrane. The membrane-bound vesicle contains proteins to be transported and is surrounded by a protective coat made up of protein subunits recruited from the cytosol. The initial budding and coating processes are controlled by a cytosolic ras-like GTP-binding protein, ADP-

30 ribosylating factor (Arf), and adapter proteins (AP). Different isoforms of both Arf and AP are involved at different sites of budding. Another small G-protein, dynamin, forms a ring complex around the neck of the forming vesicle and may provide the mechanochemical force to accomplish the final step of the budding process. The coated vesicle complex is then transported through the cytosol. During the transport process, Arf-bound GTP is hydrolyzed to GDP and the coat dissociates from the transport

35 vesicle (West, M.A. et al. (1997) J. Cell Biol. 138:1239-1254). Two different classes of coat protein

have also been identified. Clathrin coats form on the TGN and PM surfaces, whereas coatamer or COP coats form on the ER and Golgi. COP coats can further be distinguished as COPI, involved in retrograde traffic through the Golgi and from the Golgi to the ER, and COPII, involved in anterograde traffic from the ER to the Golgi (Mellman, supra). The COP coat consists of two major components, a G-protein (Arf or Sar) and coat protomer (coatamer). Coatamer is an equimolar complex of seven proteins, termed alpha-, beta-, beta', gamma-, delta-, epsilon- and zeta-COP. (Harter, C. and F.T. Wieland (1998) Proc. Natl. Acad. Sci. USA 95:11649-11654.)

#### Membrane Fusion

Transport vesicles undergo homotypic or heterotypic fusion in the secretory and endocytotic pathways. Molecules required for appropriate targeting and fusion of vesicles with their target membrane include proteins incorporated in the vesicle membrane, the target membrane, and proteins recruited from the cytosol. During budding of the vesicle from the donor compartment, an integral membrane protein, VAMP (vesicle-associated membrane protein) is incorporated into the vesicle. Soon after the vesicle uncoats, a cytosolic prenylated GTP-binding protein, Rab (a member of the Ras superfamily), is inserted into the vesicle membrane. GTP-bound Rab proteins are directed into nascent transport vesicles where they interact with VAMP. Following vesicle transport, GTPase activating proteins (GAPs) in the target membrane convert Rab proteins to the GDP-bound form. A cytosolic protein, guanine-nucleotide dissociation inhibitor (GDI) helps return GDP-bound Rab proteins to their membrane of origin. Several Rab isoforms have been identified and appear to associate with specific compartments within the cell. Rab proteins appear to play a role in mediating the function of a viral gene, Rev, which is essential for replication of HIV-1, the virus responsible for AIDS (Flavell, R.A. et al. (1996) Proc. Natl. Acad. Sci. USA 93:4421-4424).

Docking of the transport vesicle with the target membrane involves the formation of a complex between the vesicle SNAP receptor (v-SNARE), target membrane (t-) SNAREs, and certain other membrane and cytosolic proteins. Many of these other proteins have been identified although their exact functions in the docking complex remain uncertain (Tellam, J.T. et al. (1995) J. Biol. Chem. 270:5857-63; and Hata, Y. and T.C. Sudhof (1995) J. Biol. Chem. 270:13022-28). N-ethylmaleimide sensitive factor (NSF) and soluble NSF-attachment protein ( $\alpha$ -SNAP and  $\beta$ -SNAP) are two such proteins that are conserved from yeast to man and function in most intracellular membrane fusion reactions. Sec1 represents a family of yeast proteins that function at many different stages in the secretory pathway including membrane fusion. Recently, mammalian homologs of Sec1, called Munc-18 proteins, have been identified (Katagiri, H. et al. (1995) J. Biol. Chem. 270:4963-4966; Hata et al. supra).

The SNARE complex involves three SNARE molecules, one in the vesicular membrane and two in the target membrane. Synaptotagmin is an integral membrane protein in the synaptic vesicle

which associates with the t-SNARE syntaxin in the docking complex. Synaptotagmin binds calcium in a complex with negatively charged phospholipids, which allows the cytosolic SNAP protein to displace synaptotagmin from syntaxin and fusion to occur. Thus, synaptotagmin is a negative regulator of fusion in the neuron (Littleton, J.T. et al. (1993) Cell 74:1125-1134). The most abundant membrane protein of synaptic vesicles appears to be the glycoprotein synaptophysin, a 38 kDa protein with four transmembrane domains.

Specificity between a vesicle and its target is derived from the v-SNARE, t-SNAREs, and associated proteins involved. Different isoforms of SNAREs and Rabs show distinct cellular and subcellular distributions. VAMP-1/synaptobrevin, membrane-anchored synaptosome-associated protein of 25 kDa (SNAP-25), syntaxin-1, Rab3A, Rab15, and Rab23 are predominantly expressed in the brain and nervous system. Different syntaxin, VAMP, and Rab proteins are associated with distinct subcellular compartments and their vesicular carriers.

### Nuclear Transport

Transport of proteins and RNA between the nucleus and the cytoplasm occurs through nuclear pore complexes (NPCs). NPC-mediated transport occurs in both directions through the nuclear envelope. All nuclear proteins are imported from the cytoplasm, their site of synthesis. tRNA and mRNA are exported from the nucleus, their site of synthesis, to the cytoplasm, their site of function. Processing of small nuclear RNAs involves export into the cytoplasm, assembly with proteins and modifications such as hypermethylation to produce small nuclear ribonuclear proteins (snRNPs), and subsequent import of the snRNPs back into the nucleus. The assembly of ribosomes requires the initial import of ribosomal proteins from the cytoplasm, their incorporation with RNA into ribosomal subunits, and export back to the cytoplasm. (Görllich, D. and L.W. Mattaj (1996) Science 271:1513-1518.)

The transport of proteins and mRNAs across the NPC is selective, dependent on nuclear localization signals, and generally requires association with nuclear transport factors. Nuclear localization signals (NLS) consist of short stretches of amino acids enriched in basic residues. NLS are found on proteins that are targeted to the nucleus, such as the glucocorticoid receptor. The NLS is recognized by the NLS receptor, importin, which then interacts with the monomeric GTP-binding protein Ran. This NLS protein/receptor/Ran complex navigates the nuclear pore with the help of the homodimeric protein nuclear transport factor 2 (NTF2). NTF2 binds the GDP-bound form of Ran and to multiple proteins of the nuclear pore complex containing FXFG repeat motifs, such as p62. (Paschal, B. et al. (1997) J. Biol. Chem. 272:21534-21539; and Wong, D.H. et al. (1997) Mol. Cell Biol. 17:3755-3767). Some proteins are dissociated before nuclear mRNAs are transported across the NPC while others are dissociated shortly after nuclear mRNA transport across the NPC and are reimported into the nucleus.



### Disease Correlation

The etiology of numerous human diseases and disorders can be attributed to defects in the transport or secretion of proteins. For example, abnormal hormonal secretion is linked to disorders such as diabetes insipidus (vasopressin), hyper- and hypoglycemia (insulin, glucagon), Grave's disease and goiter (thyroid hormone), and Cushing's and Addison's diseases (adrenocorticotrophic hormone, ACTH). Moreover, cancer cells secrete excessive amounts of hormones or other biologically active peptides. Disorders related to excessive secretion of biologically active peptides by tumor cells include fasting hypoglycemia due to increased insulin secretion from insulinoma-islet cell tumors; hypertension due to increased epinephrine and norepinephrine secreted from pheochromocytomas of the adrenal medulla and sympathetic paraganglia; and carcinoid syndrome, which is characterized by abdominal cramps, diarrhea, and valvular heart disease caused by excessive amounts of vasoactive substances such as serotonin, bradykinin, histamine, prostaglandins, and polypeptide hormones, secreted from intestinal tumors. Biologically active peptides that are ectopically synthesized in and secreted from tumor cells include ACTH and vasopressin (lung and pancreatic cancers); parathyroid hormone (lung and bladder cancers); calcitonin (lung and breast cancers); and thyroid-stimulating hormone (medullary thyroid carcinoma). Such peptides may be useful as diagnostic markers for tumorigenesis (Schwartz, M.Z. (1997) *Semin. Pediatr. Surg.* 3:141-146; and Said, S.I. and G.R. Faloona (1975) *N. Engl. J. Med.* 293:155-160).

Defective nuclear transport may play a role in cancer. The BRCA1 protein contains three potential NLSs which interact with importin alpha, and is transported into the nucleus by the importin/NPC pathway. In breast cancer cells the BRCA1 protein is aberrantly localized in the cytoplasm. The mislocation of the BRCA1 protein in breast cancer cells may be due to a defect in the NPC nuclear import pathway (Chen, C.F. et al. (1996) *J. Biol. Chem.* 271:32863-32868).

It has been suggested that in some breast cancers, the tumor-suppressing activity of p53 is inactivated by the sequestration of the protein in the cytoplasm, away from its site of action in the cell nucleus. Cytoplasmic wild-type p53 was also found in human cervical carcinoma cell lines. (Moll, U.M. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:7262-7266; and Liang, X.H. et al. (1993) *Oncogene* 8:2645-2652.)

### Environmental Responses

Organisms respond to the environment by a number of pathways. Heat shock proteins, including hsp 70, hsp60, hsp90, and hsp 40, assist organisms in coping with heat damage to cellular proteins.

Aquaporins (AQP) are channels that transport water and, in some cases, nonionic small solutes such as urea and glycerol. Water movement is important for a number of physiological processes including renal fluid filtration, aqueous humor generation in the eye, cerebrospinal fluid production in

the brain, and appropriate hydration of the lung. Aquaporins are members of the major intrinsic protein (MIP) family of membrane transporters (King, L.S. and P. Agre (1996) *Annu. Rev. Physiol.* 58:619-648; Ishibashi, K. et al. (1997) *J. Biol. Chem.* 272:20782-20786). The study of aquaporins may have relevance to understanding edema formation and fluid balance in both normal physiology and disease states (King, *supra*). Mutations in AQP2 cause autosomal recessive nephrogenic diabetes insipidus (OMIM \*107777 Aquaporin 2; AQP2). Reduced AQP4 expression in skeletal muscle may be associated with Duchenne muscular dystrophy (Frigeri, A. et al. (1998) *J. Clin. Invest.* 102:695-703). Mutations in AQP0 cause autosomal dominant cataracts in the mouse (OMIM \*154050 Major Intrinsic Protein of Lens Fiber; MIP).

The metallothioneins (MTs) are a group of small (61 amino acids), cysteine-rich proteins that bind heavy metals such as cadmium, zinc, mercury, lead, and copper and are thought to play a role in metal detoxification or the metabolism and homeostasis of metals. Arsenite-resistance proteins have been identified in hamsters that are resistant to toxic levels of arsenite (Rossman, T.G. et al. (1997) *Mutat. Res.* 386:307-314).

Humans respond to light and odors by specific protein pathways. Proteins involved in light perception include rhodopsin, transducin, and cGMP phosphodiesterase. Proteins involved in odor perception include multiple olfactory receptors. Other proteins are important in human Circadian rhythms and responses to wounds.

#### Immunity and Host Defense

All vertebrates have developed sophisticated and complex immune systems that provide protection from viral, bacterial, fungal and parasitic infections. Included in these systems are the processes of humoral immunity, the complement cascade and the inflammatory response (Paul, W.E. (1993) Fundamental Immunology, Raven Press, Ltd., New York NY, pp.1-20).

The cellular components of the humoral immune system include six different types of leukocytes: monocytes, lymphocytes, polymorphonuclear granulocytes (consisting of neutrophils, eosinophils, and basophils) and plasma cells. Additionally, fragments of megakaryocytes, a seventh type of white blood cell in the bone marrow, occur in large numbers in the blood as platelets.

Leukocytes are formed from two stem cell lineages in bone marrow. The myeloid stem cell line produces granulocytes and monocytes and, the lymphoid stem cell produces lymphocytes.

Lymphoid cells travel to the thymus, spleen and lymph nodes, where they mature and differentiate into lymphocytes. Leukocytes are responsible for defending the body against invading pathogens. Neutrophils and monocytes attack invading bacteria, viruses, and other pathogens and destroy them by phagocytosis. Monocytes enter tissues and differentiate into macrophages which are extremely phagocytic. Lymphocytes and plasma cells are a part of the immune system which recognizes

specific foreign molecules and organisms and inactivates them, as well as signals other cells to attack

the invaders.

Granulocytes and monocytes are formed and stored in the bone marrow until needed. Megakaryocytes are produced in bone marrow, where they fragment into platelets and are released into the bloodstream. The main function of platelets is to activate the blood clotting mechanism.

- 5 Lymphocytes and plasma cells are produced in various lymphogenous organs, including the lymph nodes, spleen, thymus, and tonsils.

Both neutrophils and macrophages exhibit chemotaxis towards sites of inflammation. Tissue inflammation in response to pathogen invasion results in production of chemo-attractants for leukocytes, such as endotoxins or other bacterial products, prostaglandins, and products of leukocytes  
10 or platelets.

Basophils participate in the release of the chemicals involved in the inflammatory process. The main function of basophils is secretion of these chemicals to such a degree that they have been referred to as "unicellular endocrine glands". A distinct aspect of basophilic secretion is that the contents of granules go directly into the extracellular environment, not into vacuoles as occurs with  
15 neutrophils, eosinophils and monocytes. Basophils have receptors for the Fc fragment of immunoglobulin E (IgE) that are not present on other leukocytes. Crosslinking of membrane IgE with anti-IgE or other ligands triggers degranulation.

Eosinophils are bi- or multi-nucleated white blood cells which contain eosinophilic granules. Their plasma membrane is characterized by Ig receptors, particularly IgG and IgE. Generally,  
20 eosinophils are stored in the bone marrow until recruited for use at a site of inflammation or invasion. They have specific functions in parasitic infections and allergic reactions, and are thought to detoxify some of the substances released by mast cells and basophils which cause inflammation. Additionally, they phagocytize antigen-antibody complexes and further help prevent spread of the inflammation.

Macrophages are monocytes that have left the blood stream to settle in tissue. Once  
25 monocytes have migrated into tissues, they do not re-enter the bloodstream. The mononuclear phagocyte system is comprised of precursor cells in the bone marrow, monocytes in circulation, and macrophages in tissues. The system is capable of very fast and extensive phagocytosis. A macrophage may phagocytize over 100 bacteria, digest them and extrude residues, and then survive for many more months. Macrophages are also capable of ingesting large particles, including red  
30 blood cells and malarial parasites. They increase several-fold in size and transform into macrophages that are characteristic of the tissue they have entered, surviving in tissues for several months.

Mononuclear phagocytes are essential in defending the body against invasion by foreign pathogens, particularly intracellular microorganisms such as M. tuberculosis, listeria, leishmania and toxoplasma. Macrophages can also control the growth of tumorous cells, via both phagocytosis and  
35 secretion of hydrolytic enzymes. Another important function of macrophages is that of processing

antigen and presenting them in a biochemically modified form to lymphocytes.

The immune system responds to invading microorganisms in two major ways: antibody production and cell mediated responses. Antibodies are immunoglobulin proteins produced by B-lymphocytes which bind to specific antigens and cause inactivation or promote destruction of the antigen by other cells. Cell-mediated immune responses involve T-lymphocytes (T cells) that react with foreign antigen on the surface of infected host cells. Depending on the type of T cell, the infected cell is either killed or signals are secreted which activate macrophages and other cells to destroy the infected cell (Paul, supra).

T-lymphocytes originate in the bone marrow or liver in fetuses. Precursor cells migrate via the blood to the thymus, where they are processed to mature into T-lymphocytes. This processing is crucial because of positive and negative selection of T cells that will react with foreign antigen and not with self molecules. After processing, T cells continuously circulate in the blood and secondary lymphoid tissues, such as lymph nodes, spleen, certain epithelium-associated tissues in the gastrointestinal tract, respiratory tract and skin. When T-lymphocytes are presented with the complementary antigen, they are stimulated to proliferate and release large numbers of activated T cells into the lymph system and the blood system. These activated T cells can survive and circulate for several days. At the same time, T memory cells are created, which remain in the lymphoid tissue for months or years. Upon subsequent exposure to that specific antigen, these memory cells will respond more rapidly and with a stronger response than induced by the original antigen. This creates an "immunological memory" that can provide immunity for years.

There are two major types of T cells: cytotoxic T cells destroy infected host cells, and helper T cells activate other white blood cells via chemical signals. One class of helper cell,  $T_H1$ , activates macrophages to destroy ingested microorganisms, while another,  $T_H2$ , stimulates the production of antibodies by B cells.

Cytotoxic T cells directly attack the infected target cell. In virus-infected cells, peptides derived from viral proteins are generated by the proteasome. These peptides are transported into the ER by the transporter associated with antigen processing (TAP) (Pamer, E. and P. Cresswell (1998) Annu. Rev. Immunol. 16:323-358). Once inside the ER, the peptides bind MHC I chains, and the peptide/MHC I complex is transported to the cell surface. Receptors on the surface of T cells bind to antigen presented on cell surface MHC molecules. Once activated by binding to antigen, T cells secrete  $\gamma$ -interferon, a signal molecule that induces the expression of genes necessary for presenting viral (or other) antigens to cytotoxic T cells. Cytotoxic T cells kill the infected cell by stimulating programmed cell death.

Helper T cells constitute up to 75% of the total T cell population. They regulate the immune functions by producing a variety of lymphokines that act on other cells in the immune system and on

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bone marrow. Among these lymphokines are: interleukins-2,3,4,5,6; granulocyte-monocyte colony stimulating factor, and  $\gamma$ -interferon.

Helper T cells are required for most B cells to respond to antigen. When an activated helper cell contacts a B cell, its centrosome and Golgi apparatus become oriented toward the B cell, aiding the directing of signal molecules, such as transmembrane-bound protein called CD40 ligand, onto the B cell surface to interact with the CD40 transmembrane protein. Secreted signals also help B cells to proliferate and mature and, in some cases, to switch the class of antibody being produced.

B-lymphocytes (B cells) produce antibodies which react with specific antigenic proteins presented by pathogens. Once activated, B cells become filled with extensive rough endoplasmic reticulum and are known as plasma cells. As with T cells, interaction of B cells with antigen stimulates proliferation of only those B cells which produce antibody specific to that antigen. There are five classes of antibodies, known as immunoglobulins, which together comprise about 20% of total plasma protein. Each class mediates a characteristic biological response after antigen binding. Upon activation by specific antigen B cells switch from making membrane-bound antibody to secretion of that antibody.

Antibodies, or immunoglobulins (Ig), are the founding members of the Ig superfamily and the central components of the humoral immune response. Antibodies are either expressed on the surface of B cells or secreted by B cells into the circulation. Antibodies bind and neutralize blood-borne foreign antigens. The prototypical antibody is a tetramer consisting of two identical heavy polypeptide chains (H-chains) and two identical light polypeptide chains (L-chains) interlinked by disulfide bonds. This arrangement confers the characteristic Y-shape to antibody molecules. Antibodies are classified based on their H-chain composition. The five antibody classes, IgA, IgD, IgE, IgG and IgM, are defined by the  $\alpha$ ,  $\delta$ ,  $\epsilon$ ,  $\gamma$ , and  $\mu$  H-chain types. There are two types of L-chains,  $\kappa$  and  $\lambda$ , either of which may associate as a pair with any H-chain pair. IgG, the most common class of antibody found in the circulation, is tetrameric, while the other classes of antibodies are generally variants or multimers of this basic structure.

H-chains and L-chains each contain an N-terminal variable region and a C-terminal constant region. Both H-chains and L-chains contain repeated Ig domains. For example, a typical H-chain contains four Ig domains, three of which occur within the constant region and one of which occurs within the variable region and contributes to the formation of the antigen recognition site. Likewise, a typical L-chain contains two Ig domains, one of which occurs within the constant region and one of which occurs within the variable region. In addition, H chains such as  $\mu$  have been shown to associate with other polypeptides during differentiation of the B cell.

Antibodies can be described in terms of their two main functional domains. Antigen recognition is mediated by the Fab (antigen binding fragment) region of the antibody, while effector

functions are mediated by the Fc (crystallizable fragment) region. Binding of antibody to an antigen, such as a bacterium, triggers the destruction of the antigen by phagocytic white blood cells such as macrophages and neutrophils. These cells express surface receptors that specifically bind to the antibody Fc region and allow the phagocytic cells to engulf, ingest, and degrade the antibody-bound antigen. The Fc receptors expressed by phagocytic cells are single-pass transmembrane glycoproteins of about 300 to 400 amino acids (Sears, D.W. et al. (1990) *J. Immunol.* 144:371-378). The extracellular portion of the Fc receptor typically contains two or three Ig domains.

Diseases which cause over- or under-abundance of any one type of leukocyte usually result in the entire immune defense system becoming involved. A well-known autoimmune disease is AIDS (Acquired Immunodeficiency Syndrome) where the number of helper T cells is depleted, leaving the patient susceptible to infection by microorganisms and parasites. Another widespread medical condition attributable to the immune system is that of allergic reactions to certain antigens. Allergic reactions include: hay fever, asthma, anaphylaxis, and urticaria (hives). Leukemias are an excess production of white blood cells, to the point where a major portion of the body's metabolic resources are directed solely at proliferation of white blood cells, leaving other tissues to starve. Leukopenia or agranulocytosis occurs when the bone marrow stops producing white blood cells. This leaves the body unprotected against foreign microorganisms, including those which normally inhabit skin, mucous membranes, and gastrointestinal tract. If all white blood cell production stops completely, infection will occur within two days and death may follow only 1 to 4 days later.

Impaired phagocytosis occurs in several diseases, including monocytic leukemia, systemic lupus, and granulomatous disease. In such a situation, macrophages can phagocytize normally, but the enveloped organism is not killed. A defect in the plasma membrane enzyme which converts oxygen to lethally reactive forms results in abscess formation in liver, lungs, spleen, lymph nodes, and beneath the skin. Eosinophilia is an excess of eosinophils commonly observed in patients with allergies (hay fever, asthma), allergic reactions to drugs, rheumatoid arthritis, and cancers (Hodgkin's disease, lung, and liver cancer) (Isselbacher, K.J. et al. (1994) Harrison's Principles of Internal Medicine, McGraw-Hill, Inc., New York NY).

Host defense is further augmented by the complement system. The complement system serves as an effector system and is involved in infectious agent recognition. It can function as an independent immune network or in conjunction with other humoral immune responses. The complement system is comprised of numerous plasma and membrane proteins that act in a cascade of reaction sequences whereby one component activates the next. The result is a rapid and amplified response to infection through either an inflammatory response or increased phagocytosis.

The complement system has more than 30 protein components which can be divided into functional groupings including modified serine proteases, membrane-binding proteins and regulators

of complement activation. Activation occurs through two different pathways the classical and the alternative. Both pathways serve to destroy infectious agents through distinct triggering mechanisms that eventually merge with the involvement of the component C3.

The classical pathway requires antibody binding to infectious agent antigens. The antibodies serve to define the target and initiate the complement system cascade, culminating in the destruction of the infectious agent. In this pathway, since the antibody guides initiation of the process, the complement can be seen as an effector arm of the humoral immune system.

The alternative pathway of the complement system does not require the presence of pre-existing antibodies for targeting infectious agent destruction. Rather, this pathway, through low levels of an activated component, remains constantly primed and provides surveillance in the non-immune host to enable targeting and destruction of infectious agents. In this case foreign material triggers the cascade, thereby facilitating phagocytosis or lysis (Paul, *supra*, pp.918-919).

Another important component of host defense is the process of inflammation. Inflammatory responses are divided into four categories on the basis of pathology and include allergic inflammation, cytotoxic antibody mediated inflammation, immune complex mediated inflammation and monocyte mediated inflammation. Inflammation manifests as a combination of each of these forms with one predominating.

Allergic acute inflammation is observed in individuals wherein specific antigens stimulate IgE antibody production. Mast cells and basophils are subsequently activated by the attachment of antigen-IgE complexes, resulting in the release of cytoplasmic granule contents such as histamine. The products of activated mast cells can increase vascular permeability and constrict the smooth muscle of breathing passages, resulting in anaphylaxis or asthma. Acute inflammation is also mediated by cytotoxic antibodies and can result in the destruction of tissue through the binding of complement-fixing antibodies to cells. The responsible antibodies are of the IgG or IgM types. Resultant clinical disorders include autoimmune hemolytic anemia and thrombocytopenia as associated with systemic lupus erythematosus.

Immune complex mediated acute inflammation involves the IgG or IgM antibody types which combine with antigen to activate the complement cascade. When such immune complexes bind to neutrophils and macrophages they activate the respiratory burst to form protein- and vessel-damaging agents such as hydrogen peroxide, hydroxyl radical, hypochlorous acid, and chloramines. Clinical manifestations include rheumatoid arthritis and systemic lupus erythematosus.

In chronic inflammation or delayed-type hypersensitivity, macrophages are activated and process antigen for presentation to T cells that subsequently produce lymphokines and monokines. This type of inflammatory response is likely important for defense against intracellular parasites and certain viruses. Clinical associations include, granulomatous disease, tuberculosis, leprosy, and

sarcoidosis (Paul, W.E., supra, pp.1017-1018).

### Extracellular Information Transmission Molecules

Intercellular communication is essential for the growth and survival of multicellular organisms, and in particular, for the function of the endocrine, nervous, and immune systems. In addition, intercellular communication is critical for developmental processes such as tissue construction and organogenesis, in which cell proliferation, cell differentiation, and morphogenesis must be spatially and temporally regulated in a precise and coordinated manner. Cells communicate with one another through the secretion and uptake of diverse types of signaling molecules such as hormones, growth factors, neuropeptides, and cytokines.

#### Hormones

Hormones are signaling molecules that coordinately regulate basic physiological processes from embryogenesis throughout adulthood. These processes include metabolism, respiration, reproduction, excretion, fetal tissue differentiation and organogenesis, growth and development, homeostasis, and the stress response. Hormonal secretions and the nervous system are tightly integrated and interdependent. Hormones are secreted by endocrine glands, primarily the hypothalamus and pituitary, the thyroid and parathyroid, the pancreas, the adrenal glands, and the ovaries and testes.

The secretion of hormones into the circulation is tightly controlled. Hormones are often secreted in diurnal, pulsatile, and cyclic patterns. Hormone secretion is regulated by perturbations in blood biochemistry, by other upstream-acting hormones, by neural impulses, and by negative feedback loops. Blood hormone concentrations are constantly monitored and adjusted to maintain optimal, steady-state levels. Once secreted, hormones act only on those target cells that express specific receptors.

Most disorders of the endocrine system are caused by either hyposecretion or hypersecretion of hormones. Hyposecretion often occurs when a hormone's gland of origin is damaged or otherwise impaired. Hypersecretion often results from the proliferation of tumors derived from hormone-secreting cells. Inappropriate hormone levels may also be caused by defects in regulatory feedback loops or in the processing of hormone precursors. Endocrine malfunction may also occur when the target cell fails to respond to the hormone.

Hormones can be classified biochemically as polypeptides, steroids, eicosanoids, or amines. Polypeptides, which include diverse hormones such as insulin and growth hormone, vary in size and function and are often synthesized as inactive precursors that are processed intracellularly into mature, active forms. Amines, which include epinephrine and dopamine, are amino acid derivatives that function in neuroendocrine signaling. Steroids, which include the cholesterol-derived hormones estrogen and testosterone, function in sexual development and reproduction. Eicosanoids, which



include prostaglandins and prostacyclins, are fatty acid derivatives that function in a variety of processes. Most polypeptides and some amines are soluble in the circulation where they are highly susceptible to proteolytic degradation within seconds after their secretion. Steroids and lipids are insoluble and must be transported in the circulation by carrier proteins. The following discussion will

5 focus primarily on polypeptide hormones.

Hormones secreted by the hypothalamus and pituitary gland play a critical role in endocrine function by coordinately regulating hormonal secretions from other endocrine glands in response to neural signals. Hypothalamic hormones include thyrotropin-releasing hormone, gonadotropin-releasing hormone, somatostatin, growth-hormone releasing factor, corticotropin-releasing hormone, substance P,  
10 dopamine, and prolactin-releasing hormone. These hormones directly regulate the secretion of hormones from the anterior lobe of the pituitary. Hormones secreted by the anterior pituitary include adrenocorticotrophic hormone (ACTH), melanocyte-stimulating hormone, somatotrophic hormones such as growth hormone and prolactin, glycoprotein hormones such as thyroid-stimulating hormone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH),  $\beta$ -lipotropin, and  $\beta$ -endorphins.  
15 These hormones regulate hormonal secretions from the thyroid, pancreas, and adrenal glands, and act directly on the reproductive organs to stimulate ovulation and spermatogenesis. The posterior pituitary synthesizes and secretes antidiuretic hormone (ADH, vasopressin) and oxytocin.

Disorders of the hypothalamus and pituitary often result from lesions such as primary brain tumors, adenomas, infarction associated with pregnancy, hypophysectomy, aneurysms, vascular  
20 malformations, thrombosis, infections, immunological disorders, and complications due to head trauma. Such disorders have profound effects on the function of other endocrine glands. Disorders associated with hypopituitarism include hypogonadism, Sheehan syndrome, diabetes insipidus, Kallman's disease, Hand-Schuller-Christian disease, Letterer-Siwe disease, sarcoidosis, empty sella syndrome, and dwarfism. Disorders associated with hyperpituitarism include acromegaly, gigantism, and syndrome of  
25 inappropriate ADH secretion (SIADH), often caused by benign adenomas.

Hormones secreted by the thyroid and parathyroid primarily control metabolic rates and the regulation of serum calcium levels, respectively. Thyroid hormones include calcitonin, somatostatin, and thyroid hormone. The parathyroid secretes parathyroid hormone. Disorders associated with hypothyroidism include goiter, myxedema, acute thyroiditis associated with bacterial infection,  
30 subacute thyroiditis associated with viral infection, autoimmune thyroiditis (Hashimoto's disease), and cretinism. Disorders associated with hyperthyroidism include thyrotoxicosis and its various forms, Grave's disease, pretibial myxedema, toxic multinodular goiter, thyroid carcinoma, and Plummer's disease. Disorders associated with hyperparathyroidism include Conn disease (chronic hypercalcemia) leading to bone resorption and parathyroid hyperplasia.

35 Hormones secreted by the pancreas regulate blood glucose levels by modulating the rates of

carbohydrate, fat, and protein metabolism. Pancreatic hormones include insulin, glucagon, amylin,  $\gamma$ -aminobutyric acid, gastrin, somatostatin, and pancreatic polypeptide. The principal disorder associated with pancreatic dysfunction is diabetes mellitus caused by insufficient insulin activity. Diabetes mellitus is generally classified as either Type I (insulin-dependent, juvenile diabetes) or Type II (non-insulin-dependent, adult diabetes). The treatment of both forms by insulin replacement therapy is well known. Diabetes mellitus often leads to acute complications such as hypoglycemia (insulin shock), coma, diabetic ketoacidosis, lactic acidosis, and chronic complications leading to disorders of the eye, kidney, skin, bone, joint, cardiovascular system, nervous system, and to decreased resistance to infection.

The anatomy, physiology, and diseases related to hormonal function are reviewed in McCance, K.L. and S.E. Huether (1994) Pathophysiology: The Biological Basis for Disease in Adults and Children, Mosby-Year Book, Inc., St. Louis MO; Greenspan, F.S. and J.D. Baxter (1994) Basic and Clinical Endocrinology, Appleton and Lange, East Norwalk CT.

#### Growth Factors

Growth factors are secreted proteins that mediate intercellular communication. Unlike hormones, which travel great distances via the circulatory system, most growth factors are primarily local mediators that act on neighboring cells. Most growth factors contain a hydrophobic N-terminal signal peptide sequence which directs the growth factor into the secretory pathway. Most growth factors also undergo post-translational modifications within the secretory pathway. These modifications can include proteolysis, glycosylation, phosphorylation, and intramolecular disulfide bond formation. Once secreted, growth factors bind to specific receptors on the surfaces of neighboring target cells, and the bound receptors trigger intracellular signal transduction pathways. These signal transduction pathways elicit specific cellular responses in the target cells. These responses can include the modulation of gene expression and the stimulation or inhibition of cell division, cell differentiation, and cell motility.

Growth factors fall into at least two broad and overlapping classes. The broadest class includes the large polypeptide growth factors, which are wide-ranging in their effects. These factors include epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor- $\beta$  (TGF- $\beta$ ), insulin-like growth factor (IGF), nerve growth factor (NGF), and platelet-derived growth factor (PDGF), each defining a family of numerous related factors. The large polypeptide growth factors, with the exception of NGF, act as mitogens on diverse cell types to stimulate wound healing, bone synthesis and remodeling, extracellular matrix synthesis, and proliferation of epithelial, epidermal, and connective tissues. Members of the TGF- $\beta$ , EGF, and FGF families also function as inductive signals in the differentiation of embryonic tissue. NGF functions specifically as a neurotrophic factor, promoting neuronal growth and differentiation.

Another class of growth factors includes the hematopoietic growth factors, which are narrow in their target specificity. These factors stimulate the proliferation and differentiation of blood cells such as B-lymphocytes, T-lymphocytes, erythrocytes, platelets, eosinophils, basophils, neutrophils, macrophages, and their stem cell precursors. These factors include the colony-stimulating factors (G-CSF, M-CSF, GM-CSF, and CSF1-3), erythropoietin, and the cytokines. The cytokines are specialized hematopoietic factors secreted by cells of the immune system and are discussed in detail below.

Growth factors play critical roles in neoplastic transformation of cells *in vitro* and in tumor progression *in vivo*. Overexpression of the large polypeptide growth factors promotes the proliferation and transformation of cells in culture. Inappropriate expression of these growth factors by tumor cells *in vivo* may contribute to tumor vascularization and metastasis. Inappropriate activity of hematopoietic growth factors can result in anemias, leukemias, and lymphomas. Moreover, growth factors are both structurally and functionally related to oncoproteins, the potentially cancer-causing products of proto-oncogenes. Certain FGF and PDGF family members are themselves homologous to oncoproteins, whereas receptors for some members of the EGF, NGF, and FGF families are encoded by proto-oncogenes. Growth factors also affect the transcriptional regulation of both proto-oncogenes and oncosuppressor genes (Pimentel, E. (1994) Handbook of Growth Factors, CRC Press, Ann Arbor MI; McKay, I. and I. Leigh, eds. (1993) Growth Factors: A Practical Approach, Oxford University Press, New York NY; Habenicht, A., ed. (1990) Growth Factors, Differentiation Factors, and Cytokines, Springer-Verlag, New York NY).

In addition, some of the large polypeptide growth factors play crucial roles in the induction of the primordial germ layers in the developing embryo. This induction ultimately results in the formation of the embryonic mesoderm, ectoderm, and endoderm which in turn provide the framework for the entire adult body plan. Disruption of this inductive process would be catastrophic to embryonic development.

#### Small Peptide Factors - Neuropeptides and Vasomediators

Neuropeptides and vasomediators (NP/VM) comprise a family of small peptide factors, typically of 20 amino acids or less. These factors generally function in neuronal excitation and inhibition of vasoconstriction/vasodilation, muscle contraction, and hormonal secretions from the brain and other endocrine tissues. Included in this family are neuropeptides and neuropeptide hormones such as bombesin, neuropeptide Y, neurotensin, neuromedin N, melanocortins, opioids, galanin, somatostatin, tachykinins, urotensin II and related peptides involved in smooth muscle stimulation, vasopressin, vasoactive intestinal peptide, and circulatory system-borne signaling molecules such as angiotensin, complement, calcitonin, endothelins, formyl-methionyl peptides, glucagon, cholecystokinin, gastrin, and many of the peptide hormones discussed above. NP/VMs can transduce signals directly, modulate the activity or release of other neurotransmitters and hormones, and

act as catalytic enzymes in signaling cascades. The effects of NP/VMs range from extremely brief to long-lasting. (Reviewed in Martin, C.R. et al. (1985) Endocrine Physiology, Oxford University Press, New York NY, pp. 57-62.)

### Cytokines

5 Cytokines comprise a family of signaling molecules that modulate the immune system and the inflammatory response. Cytokines are usually secreted by leukocytes, or white blood cells, in response to injury or infection. Cytokines function as growth and differentiation factors that act primarily on cells of the immune system such as B- and T-lymphocytes, monocytes, macrophages, and granulocytes. Like other signaling molecules, cytokines bind to specific plasma membrane receptors and trigger  
10 intracellular signal transduction pathways which alter gene expression patterns. There is considerable potential for the use of cytokines in the treatment of inflammation and immune system disorders.

Cytokine structure and function have been extensively characterized in vitro. Most cytokines are small polypeptides of about 30 kilodaltons or less. Over 50 cytokines have been identified from human and rodent sources. Examples of cytokine subfamilies include the interferons (IFN- $\alpha$ , - $\beta$ , and -  
15  $\gamma$ ), the interleukins (IL1-IL13), the tumor necrosis factors (TNF- $\alpha$  and - $\beta$ ), and the chemokines. Many cytokines have been produced using recombinant DNA techniques, and the activities of individual cytokines have been determined in vitro. These activities include regulation of leukocyte proliferation, differentiation, and motility.

The activity of an individual cytokine in vitro may not reflect the full scope of that cytokine's  
20 activity in vivo. Cytokines are not expressed individually in vivo but are instead expressed in combination with a multitude of other cytokines when the organism is challenged with a stimulus. Together, these cytokines collectively modulate the immune response in a manner appropriate for that particular stimulus. Therefore, the physiological activity of a cytokine is determined by the stimulus itself and by complex interactive networks among co-expressed cytokines which may demonstrate both  
25 synergistic and antagonistic relationships.

Chemokines comprise a cytokine subfamily with over 30 members. (Reviewed in Wells, T. N.C. and M.C. Peitsch (1997) J. Leukoc. Biol. 61:545-550.) Chemokines were initially identified as chemotactic proteins that recruit monocytes and macrophages to sites of inflammation. Recent evidence indicates that chemokines may also play key roles in hematopoiesis and HIV-1 infection. Chemokines  
30 are small proteins which range from about 6-15 kilodaltons in molecular weight. Chemokines are further classified as C, CC, CXC, or CX<sub>2</sub>C based on the number and position of critical cysteine residues. The CC chemokines, for example, each contain a conserved motif consisting of two consecutive cysteines followed by two additional cysteines which occur downstream at 24- and 16-residue intervals, respectively (ExpASY PROSITE database, documents PS00472 and PDOC00434).  
35 The presence and spacing of these four cysteine residues are highly conserved, whereas the intervening

residues diverge significantly. However, a conserved tyrosine located about 15 residues downstream of the cysteine doublet seems to be important for chemotactic activity. Most of the human genes encoding CC chemokines are clustered on chromosome 17, although there are a few examples of CC chemokine genes that map elsewhere. Other chemokines include lymphotactin (C chemokine); macrophage chemotactic and activating factor (MCAF/MCP-1; CC chemokine); platelet factor 4 and IL-8 (CXC chemokines); and fractalkine and neurotractin (CX<sub>3</sub>C chemokines). (Reviewed in Luster, A.D. (1998) N. Engl. J. Med. 338:436-445.)

### Receptor Molecules

SEQ ID NO:6 and SEQ ID NO:7 encode, for example, receptor molecules.

The term receptor describes proteins that specifically recognize other molecules. The category is broad and includes proteins with a variety of functions. The bulk of receptors are cell surface proteins which bind extracellular ligands and produce cellular responses in the areas of growth, differentiation, endocytosis, and immune response. Other receptors facilitate the selective transport of proteins out of the endoplasmic reticulum and localize enzymes to particular locations in the cell. The term may also be applied to proteins which act as receptors for ligands with known or unknown chemical composition and which interact with other cellular components. For example, the steroid hormone receptors bind to and regulate transcription of DNA.

Regulation of cell proliferation, differentiation, and migration is important for the formation and function of tissues. Regulatory proteins such as growth factors coordinately control these cellular processes and act as mediators in cell-cell signaling pathways. Growth factors are secreted proteins that bind to specific cell-surface receptors on target cells. The bound receptors trigger intracellular signal transduction pathways which activate various downstream effectors that regulate gene expression, cell division, cell differentiation, cell motility, and other cellular processes.

Cell surface receptors are typically integral plasma membrane proteins. These receptors recognize hormones such as catecholamines; peptide hormones; growth and differentiation factors; small peptide factors such as thyrotropin-releasing hormone; galanin, somatostatin, and tachykinins; and circulatory system-borne signaling molecules. Cell surface receptors on immune system cells recognize antigens, antibodies, and major histocompatibility complex (MHC)-bound peptides. Other cell surface receptors bind ligands to be internalized by the cell. This receptor-mediated endocytosis functions in the uptake of low density lipoproteins (LDL), transferrin, glucose- or mannose-terminal glycoproteins, galactose-terminal glycoproteins, immunoglobulins, phosphotigogenins, fibrin, proteinase-inhibitor complexes, plasminogen activators, and thrombospondin (Lodish, H. et al. (1995) Molecular Cell Biology, Scientific American Books, New York NY, p. 723; Mikhailenko, I. et al. (1997) J. Biol. Chem. 272:6784-6791).

### Receptor Protein Kinases

Many growth factor receptors, including receptors for epidermal growth factor, platelet-derived growth factor, fibroblast growth factor, as well as the growth modulator  $\alpha$ -thrombin, contain intrinsic protein kinase activities. When growth factor binds to the receptor, it triggers the autophosphorylation of a serine, threonine, or tyrosine residue on the receptor. These phosphorylated sites are recognition sites for the binding of other cytoplasmic signaling proteins. These proteins participate in signaling pathways that eventually link the initial receptor activation at the cell surface to the activation of a specific intracellular target molecule. In the case of tyrosine residue autophosphorylation, these signaling proteins contain a common domain referred to as a Src homology (SH) domain. SH2 domains and SH3 domains are found in phospholipase C- $\gamma$ , PI-3-K p85 regulatory subunit, Ras-GTPase activating protein, and pp60<sup>c-src</sup> (Lowenstein, E.J. et al. (1992) Cell 70:431-442). The cytokine family of receptors share a different common binding domain and include transmembrane receptors for growth hormone (GH), interleukins, erythropoietin, and prolactin.

Other receptors and second messenger-binding proteins have intrinsic serine/threonine protein kinase activity. These include activin/TGF- $\beta$ /BMP-superfamily receptors, calcium- and diacylglycerol-activated/phospholipid-dependant protein kinase (PK-C), and RNA-dependant protein kinase (PK-R). In addition, other serine/threonine protein kinases, including nematode Twitchin, have fibronectin-like, immunoglobulin C2-like domains.

### G-Protein Coupled Receptors

G-protein coupled receptors (GPCRs) are integral membrane proteins characterized by the presence of seven hydrophobic transmembrane domains which span the plasma membrane and form a bundle of antiparallel alpha ( $\alpha$ ) helices. These proteins range in size from under 400 to over 1000 amino acids (Strosberg, A.D. (1991) Eur. J. Biochem. 196:1-10; Coughlin, S.R. (1994) Curr. Opin. Cell Biol. 6:191-197). The amino-terminus of the GPCR is extracellular, of variable length and often glycosylated; the carboxy-terminus is cytoplasmic and generally phosphorylated. Extracellular loops of the GPCR alternate with intracellular loops and link the transmembrane domains. The most conserved domains of GPCRs are the transmembrane domains and the first two cytoplasmic loops. The transmembrane domains account for structural and functional features of the receptor. In most cases, the bundle of  $\alpha$  helices forms a binding pocket. In addition, the extracellular N-terminal segment or one or more of the three extracellular loops may also participate in ligand binding. Ligand binding activates the receptor by inducing a conformational change in intracellular portions of the receptor. The activated receptor, in turn, interacts with an intracellular heterotrimeric guanine nucleotide binding (G) protein complex which mediates further intracellular signaling activities, generally the production of second messengers such as cyclic AMP (cAMP), phospholipase C, inositol triphosphate, or interactions with ion channel proteins (Baldwin, J.M. (1994) Curr. Opin. Cell Biol. 6:180-190).

GPCRs include those for acetylcholine, adenosine, epinephrine and norepinephrine, bombesin, bradykinin, chemokines, dopamine, endothelin,  $\gamma$ -aminobutyric acid (GABA), follicle-stimulating hormone (FSH), glutamate, gonadotropin-releasing hormone (GnRH), hepatocyte growth factor, histamine, leukotrienes, melanocortins, neuropeptide Y, opioid peptides, opsins, prostanoids, serotonin, somatostatin, tachykinins, thrombin, thyrotropin-releasing hormone (TRH), vasoactive intestinal polypeptide family, vasopressin and oxytocin, and orphan receptors.

GPCR mutations, which may cause loss of function or constitutive activation, have been associated with numerous human diseases (Coughlin, supra). For instance, retinitis pigmentosa may arise from mutations in the rhodopsin gene. Rhodopsin is the retinal photoreceptor which is located within the discs of the eye rod cell. Parma, J. et al. (1993, Nature 365:649-651) report that somatic activating mutations in the thyrotropin receptor cause hyperfunctioning thyroid adenomas and suggest that certain GPCRs susceptible to constitutive activation may behave as protooncogenes.

#### Nuclear Receptors

Nuclear receptors bind small molecules such as hormones or second messengers, leading to increased receptor-binding affinity to specific chromosomal DNA elements. In addition the affinity for other nuclear proteins may also be altered. Such binding and protein-protein interactions may regulate and modulate gene expression. Examples of such receptors include the steroid hormone receptors family, the retinoic acid receptors family, and the thyroid hormone receptors family.

#### Ligand-Gated Receptor Ion Channels

Ligand-gated receptor ion channels fall into two categories. The first category, extracellular ligand-gated receptor ion channels (ELGs), rapidly transduce neurotransmitter-binding events into electrical signals, such as fast synaptic neurotransmission. ELG function is regulated by post-translational modification. The second category, intracellular ligand-gated receptor ion channels (ILGs), are activated by many intracellular second messengers and do not require post-translational modification(s) to effect a channel-opening response.

ELGs depolarize excitable cells to the threshold of action potential generation. In non-excitable cells, ELGs permit a limited calcium ion-influx during the presence of agonist. ELGs include channels directly gated by neurotransmitters such as acetylcholine, L-glutamate, glycine, ATP, serotonin, GABA, and histamine. ELG genes encode proteins having strong structural and functional similarities. ILGs are encoded by distinct and unrelated gene families and include receptors for cAMP, cGMP, calcium ions, ATP, and metabolites of arachidonic acid.

#### Macrophage Scavenger Receptors

Macrophage scavenger receptors with broad ligand specificity may participate in the binding of low density lipoproteins (LDL) and foreign antigens. Scavenger receptors types I and II are trimeric membrane proteins with each subunit containing a small N-terminal intracellular domain, a

transmembrane domain, a large extracellular domain, and a C-terminal cysteine-rich domain. The extracellular domain contains a short spacer domain, an  $\alpha$ -helical coiled-coil domain, and a triple helical collagenous domain. These receptors have been shown to bind a spectrum of ligands, including chemically modified lipoproteins and albumin, polyribonucleotides, polysaccharides, phospholipids, and asbestos (Matsumoto, A. et al. (1990) Proc. Natl. Acad. Sci. USA 87:9133-9137; Eliomaa, O. et al. (1995) Cell 80:603-609). The scavenger receptors are thought to play a key role in atherogenesis by mediating uptake of modified LDL in arterial walls, and in host defense by binding bacterial endotoxins, bacteria, and protozoa.

#### T-Cell Receptors

T cells play a dual role in the immune system as effectors and regulators, coupling antigen recognition with the transmission of signals that induce cell death in infected cells and stimulate proliferation of other immune cells. Although a population of T cells can recognize a wide range of different antigens, an individual T cell can only recognize a single antigen and only when it is presented to the T cell receptor (TCR) as a peptide complexed with a major histocompatibility molecule (MHC) on the surface of an antigen presenting cell. The TCR on most T cells consists of immunoglobulin-like integral membrane glycoproteins containing two polypeptide subunits,  $\alpha$  and  $\beta$ , of similar molecular weight. Both TCR subunits have an extracellular domain containing both variable and constant regions, a transmembrane domain that traverses the membrane once, and a short intracellular domain (Saito, H. et al. (1984) Nature 309:757-762). The genes for the TCR subunits are constructed through somatic rearrangement of different gene segments. Interaction of antigen in the proper MHC context with the TCR initiates signaling cascades that induce the proliferation, maturation, and function of cellular components of the immune system (Weiss, A. (1991) Annu. Rev. Genet. 25:487-510). Rearrangements in TCR genes and alterations in TCR expression have been noted in lymphomas, leukemias, autoimmune disorders, and immunodeficiency disorders (Aisenberg, A.C. et al. (1985) N. Engl. J. Med. 313:529-533; Weiss, supra).

#### Intracellular Signaling Molecules

SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12 encode, for example, intracellular signaling molecules.

Intracellular signaling is the general process by which cells respond to extracellular signals (hormones, neurotransmitters, growth and differentiation factors, etc.) through a cascade of biochemical reactions that begins with the binding of a signaling molecule to a cell membrane receptor and ends with the activation of an intracellular target molecule. Intermediate steps in the process involve the activation of various cytoplasmic proteins by phosphorylation via protein kinases, and their deactivation by protein phosphatases, and the eventual translocation of some of these



activated proteins to the cell nucleus where the transcription of specific genes is triggered. The intracellular signaling process regulates all types of cell functions including cell proliferation, cell differentiation, and gene transcription, and involves a diversity of molecules including protein kinases and phosphatases, and second messenger molecules, such as cyclic nucleotides, calcium-calmodulin, inositol, and various mitogens, that regulate protein phosphorylation.

#### Protein Phosphorylation

Protein kinases and phosphatases play a key role in the intracellular signaling process by controlling the phosphorylation and activation of various signaling proteins. The high energy phosphate for this reaction is generally transferred from the adenosine triphosphate molecule (ATP) to a particular protein by a protein kinase and removed from that protein by a protein phosphatase. Protein kinases are roughly divided into two groups: those that phosphorylate tyrosine residues (protein tyrosine kinases, PTK) and those that phosphorylate serine or threonine residues (serine/threonine kinases, STK). A few protein kinases have dual specificity for serine/threonine and tyrosine residues. Almost all kinases contain a conserved 250-300 amino acid catalytic domain containing specific residues and sequence motifs characteristic of the kinase family (Hardie, G. and S. Hanks (1995) The Protein Kinase Facts Books, Vol I:7-20, Academic Press, San Diego CA).

STKs include the second messenger dependent protein kinases such as the cyclic-AMP dependent protein kinases (PKA), involved in mediating hormone-induced cellular responses; calcium-calmodulin (CaM) dependent protein kinases, involved in regulation of smooth muscle contraction, glycogen breakdown, and neurotransmission; and the mitogen-activated protein kinases (MAP) which mediate signal transduction from the cell surface to the nucleus via phosphorylation cascades. Altered PKA expression is implicated in a variety of disorders and diseases including cancer, thyroid disorders, diabetes, atherosclerosis, and cardiovascular disease (Isselbacher, K.J. et al. (1994) Harrison's Principles of Internal Medicine McGraw-Hill, New York NY, pp. 416-431, 1887).

PTKs are divided into transmembrane, receptor PTKs and nontransmembrane, non-receptor PTKs. Transmembrane PTKs are receptors for most growth factors. Non-receptor PTKs lack transmembrane regions and, instead, form complexes with the intracellular regions of cell surface receptors. Receptors that function through non-receptor PTKs include those for cytokines and hormones (growth hormone and prolactin) and antigen-specific receptors on T and B lymphocytes. Many of these PTKs were first identified as the products of mutant oncogenes in cancer cells in which their activation was no longer subject to normal cellular controls. In fact, about one third of the known oncogenes encode PTKs, and it is well known that cellular transformation (oncogenesis) is often accompanied by increased tyrosine phosphorylation activity (Charbonneau, H. and N.K. Tonks (1992) *Annu. Rev. Cell Biol.* 8:463-493).

An additional family of protein kinases previously thought to exist only in procaryotes is the

histidine protein kinase family (HPK). HPKs bear little homology with mammalian STKs or PTKs but have distinctive sequence motifs of their own (Davie, J.R. et al. (1995) J. Biol. Chem. 270:19861-19867). A histidine residue in the N-terminal half of the molecule (region I) is an autophosphorylation site. Three additional motifs located in the C-terminal half of the molecule include an invariant asparagine residue in region II and two glycine-rich loops characteristic of nucleotide binding domains in regions III and IV. Recently a branched chain alpha-ketoacid dehydrogenase kinase has been found with characteristics of HPK in rat (Davie, supra).

Protein phosphatases regulate the effects of protein kinases by removing phosphate groups from molecules previously activated by kinases. The two principal categories of protein phosphatases are the protein (serine/threonine) phosphatases (PPs) and the protein tyrosine phosphatases (PTPs). PPs dephosphorylate phosphoserine/threonine residues and are important regulators of many cAMP-mediated hormone responses (Cohen, P. (1989) Annu. Rev. Biochem. 58:453-508). PTPs reverse the effects of protein tyrosine kinases and play a significant role in cell cycle and cell signaling processes (Charbonneau, supra). As previously noted, many PTKs are encoded by oncogenes, and oncogenesis is often accompanied by increased tyrosine phosphorylation activity. It is therefore possible that PTPs may prevent or reverse cell transformation and the growth of various cancers by controlling the levels of tyrosine phosphorylation in cells. This hypothesis is supported by studies showing that overexpression of PTPs can suppress transformation in cells, and that specific inhibition of PTPs can enhance cell transformation (Charbonneau, supra).

#### Phospholipid and Inositol-Phosphate Signaling

Inositol phospholipids (phosphoinositides) are involved in an intracellular signaling pathway that begins with binding of a signaling molecule to a G-protein linked receptor in the plasma membrane. This leads to the phosphorylation of phosphatidylinositol (PI) residues on the inner side of the plasma membrane to the biphosphate state (PIP<sub>2</sub>) by inositol kinases. Simultaneously, the G-protein linked receptor binding stimulates a trimeric G-protein which in turn activates a phosphoinositide-specific phospholipase C- $\beta$ . Phospholipase C- $\beta$  then cleaves PIP<sub>2</sub> into two products, inositol triphosphate (IP<sub>3</sub>) and diacylglycerol. These two products act as mediators for separate signaling events. IP<sub>3</sub> diffuses through the plasma membrane to induce calcium release from the endoplasmic reticulum (ER), while diacylglycerol remains in the membrane and helps activate protein kinase C, an STK that phosphorylates selected proteins in the target cell. The calcium response initiated by IP<sub>3</sub> is terminated by the dephosphorylation of IP<sub>3</sub> by specific inositol phosphatases. Cellular responses that are mediated by this pathway are glycogen breakdown in the liver in response to vasopressin, smooth muscle contraction in response to acetylcholine, and thrombin-induced platelet aggregation.

#### Cyclic Nucleotide Signaling

Cyclic nucleotides (cAMP and cGMP) function as intracellular second messengers to transduce a variety of extracellular signals including hormones, light, and neurotransmitters. In particular, cyclic-AMP dependent protein kinases (PKA) are thought to account for all of the effects of cAMP in most mammalian cells, including various hormone-induced cellular responses. Visual excitation and the phototransmission of light signals in the eye is controlled by cyclic-GMP regulated,  $Ca^{2+}$ -specific channels. Because of the importance of cellular levels of cyclic nucleotides in mediating these various responses, regulating the synthesis and breakdown of cyclic nucleotides is an important matter. Thus adenylyl cyclase, which synthesizes cAMP from AMP, is activated to increase cAMP levels in muscle by binding of adrenaline to  $\beta$ -adrenergic receptors, while activation of guanylate cyclase and increased cGMP levels in photoreceptors leads to reopening of the  $Ca^{2+}$ -specific channels and recovery of the dark state in the eye. In contrast, hydrolysis of cyclic nucleotides by cAMP and cGMP-specific phosphodiesterases (PDEs) produces the opposite of these and other effects mediated by increased cyclic nucleotide levels. PDEs appear to be particularly important in the regulation of cyclic nucleotides, considering the diversity found in this family of proteins. At least seven families of mammalian PDEs (PDE1-7) have been identified based on substrate specificity and affinity, sensitivity to cofactors, and sensitivity to inhibitory drugs (Beavo, J.A. (1995) *Physiological Reviews* 75:725-48). PDE inhibitors have been found to be particularly useful in treating various clinical disorders. Rolipram, a specific inhibitor of PDE4, has been used in the treatment of depression, and similar inhibitors are undergoing evaluation as anti-inflammatory agents. Theophylline is a nonspecific PDE inhibitor used in the treatment of bronchial asthma and other respiratory diseases (Banner, K.H. and C.P. Page (1995) *Eur. Respir. J.* 8:996-1000).

#### G-Protein Signaling

Guanine nucleotide binding proteins (G-proteins) are critical mediators of signal transduction between a particular class of extracellular receptors, the G-protein coupled receptors (GPCR), and intracellular second messengers such as cAMP and  $Ca^{2+}$ . G-proteins are linked to the cytosolic side of a GPCR such that activation of the GPCR by ligand binding stimulates binding of the G-protein to GTP, inducing an "active" state in the G-protein. In the active state, the G-protein acts as a signal to trigger other events in the cell such as the increase of cAMP levels or the release of  $Ca^{2+}$  into the cytosol from the ER, which, in turn, regulate phosphorylation and activation of other intracellular proteins. Recycling of the G-protein to the inactive state involves hydrolysis of the bound GTP to GDP by a GTPase activity in the G-protein. (See Alberts, B. et al. (1994) Molecular Biology of the Cell. Garland Publishing, Inc., New York NY, pp.734-759.) Two structurally distinct classes of G-proteins are recognized: heterotrimeric G-proteins, consisting of three different subunits, and monomeric, low molecular weight (LMW), G-proteins consisting of a single polypeptide chain.

The three polypeptide subunits of heterotrimeric G-proteins are the  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits. The

$\alpha$  subunit binds and hydrolyzes GTP. The  $\beta$  and  $\gamma$  subunits form a tight complex that anchors the protein to the inner side of the plasma membrane. The  $\beta$  subunits, also known as G- $\beta$  proteins or  $\beta$  transducins, contain seven tandem repeats of the WD-repeat sequence motif, a motif found in many proteins with regulatory functions. Mutations and variant expression of  $\beta$  transducin proteins are

- 5 linked with various disorders (Neer, E.J. et al. (1994) *Nature* 371:297-300; Margottin, F. et al. (1998) *Mol. Cell* 1:565-574).

LMW GTP-proteins are GTPases which regulate cell growth, cell cycle control, protein secretion, and intracellular vesicle interaction. They consist of single polypeptides which, like the  $\alpha$  subunit of the heterotrimeric G-proteins, are able to bind and hydrolyze GTP, thus cycling between an

10 inactive and an active state. At least sixty members of the LMW G-protein superfamily have been identified and are currently grouped into the six subfamilies of ras, rho, arf, sar1, ran, and rab. Activated ras genes were initially found in human cancers, and subsequent studies confirmed that ras function is critical in determining whether cells continue to grow or become differentiated. Other members of the LMW G-protein superfamily have roles in signal transduction that vary with the

15 function of the activated genes and the locations of the G-proteins.

Guanine nucleotide exchange factors regulate the activities of LMW G-proteins by determining whether GTP or GDP is bound. GTPase-activating protein (GAP) binds to GTP-ras and induces it to hydrolyze GTP to GDP. In contrast, guanine nucleotide releasing protein (GNRP) binds to GDP-ras and induces the release of GDP and the binding of GTP.

20 Other regulators of G-protein signaling (RGS) also exist that act primarily by negatively regulating the G-protein pathway by an unknown mechanism (Druey, K.M. et al. (1996) *Nature* 379:742-746). Some 15 members of the RGS family have been identified. RGS family members are related structurally through similarities in an approximately 120 amino acid region termed the RGS domain and functionally by their ability to inhibit the interleukin (cytokine) induction of MAP kinase

25 in cultured mammalian 293T cells (Druey, *supra*).

#### Calcium Signaling Molecules

$\text{Ca}^{+2}$  is another second messenger molecule that is even more widely used as an intracellular mediator than cAMP. Two pathways exist by which  $\text{Ca}^{+2}$  can enter the cytosol in response to extracellular signals: One pathway acts primarily in nerve signal transduction where  $\text{Ca}^{+2}$  enters a

30 nerve terminal through a voltage-gated  $\text{Ca}^{+2}$  channel. The second is a more ubiquitous pathway in which  $\text{Ca}^{+2}$  is released from the ER into the cytosol in response to binding of an extracellular signaling molecule to a receptor.  $\text{Ca}^{+2}$  directly activates regulatory enzymes, such as protein kinase C, which trigger signal transduction pathways.  $\text{Ca}^{+2}$  also binds to specific  $\text{Ca}^{+2}$ -binding proteins (CBPs) such as calmodulin (CaM) which then activate multiple target proteins in the cell including enzymes,

35 membrane transport pumps, and ion channels. CaM interactions are involved in a multitude of cellular

processes including, but not limited to, gene regulation, DNA synthesis, cell cycle progression, mitosis, cytokinesis, cytoskeletal organization, muscle contraction, signal transduction, ion homeostasis, exocytosis, and metabolic regulation (Celio, M.R. et al. (1996) Guidebook to Calcium-binding Proteins, Oxford University Press, Oxford, UK, pp. 15-20). Some CBPs can serve as a storage depot for  $\text{Ca}^{2+}$  in an inactive state. Calsequestrin is one such CBP that is expressed in isoforms specific to cardiac muscle and skeletal muscle. It is suggested that calsequestrin binds  $\text{Ca}^{2+}$  in a rapidly exchangeable state that is released during  $\text{Ca}^{2+}$ -signaling conditions (Celio, M.R. et al. (1996) Guidebook to Calcium-binding Proteins, Oxford University Press, New York NY, pp. 222-224).

#### 10 Cyclins

Cell division is the fundamental process by which all living things grow and reproduce. In most organisms, the cell cycle consists of three principle steps; interphase, mitosis, and cytokinesis. Interphase, involves preparations for cell division, replication of the DNA and production of essential proteins. In mitosis, the nuclear material is divided and separates to opposite sides of the cell. Cytokinesis is the final division and fission of the cell cytoplasm to produce the daughter cells.

The entry and exit of a cell from mitosis is regulated by the synthesis and destruction of a family of activating proteins called cyclins. Cyclins act by binding to and activating a group of cyclin-dependent protein kinases (Cdks) which then phosphorylate and activate selected proteins involved in the mitotic process. Several types of cyclins exist. (Ciechanover, A. (1994) Cell 79:13-21.) Two principle types are mitotic cyclin, or cyclin B, which controls entry of the cell into mitosis, and G1 cyclin, which controls events that drive the cell out of mitosis.

#### 20 Signal Complex Scaffolding Proteins

Certain proteins in intracellular signaling pathways serve to link or cluster other proteins involved in the signaling cascade. A conserved protein domain called the PDZ domain has been identified in various membrane-associated signaling proteins. This domain has been implicated in receptor and ion channel clustering and in the targeting of multiprotein signaling complexes to specialized functional regions of the cytosolic face of the plasma membrane. (For a review of PDZ domain-containing proteins, see Ponting, C.P. et al. (1997) Bioessays 19:469-479.) A large proportion of PDZ domains are found in the eukaryotic MAGUK (membrane-associated guanylate kinase) protein family, members of which bind to the intracellular domains of receptors and channels. However, PDZ domains are also found in diverse membrane-localized proteins such as protein tyrosine phosphatases, serine/threonine kinases, G-protein cofactors, and synapse-associated proteins such as syntrophins and neuronal nitric oxide synthase (nNOS). Generally, about one to three PDZ domains are found in a given protein, although up to nine PDZ domains have been identified in a single protein.

## Membrane Transport Molecules

SEQ ID NO:13 encodes, for example, a membrane transport molecule.

The plasma membrane acts as a barrier to most molecules. Transport between the cytoplasm and the extracellular environment, and between the cytoplasm and luminal spaces of cellular organelles requires specific transport proteins. Each transport protein carries a particular class of molecule, such as ions, sugars, or amino acids, and often is specific to a certain molecular species of the class. A variety of human inherited diseases are caused by a mutation in a transport protein. For example, cystinuria is an inherited disease that results from the inability to transport cystine, the disulfide-linked dimer of cysteine, from the urine into the blood. Accumulation of cystine in the urine leads to the formation of cystine stones in the kidneys.

Transport proteins are multi-pass transmembrane proteins, which either actively transport molecules across the membrane or passively allow them to cross. Active transport involves directional pumping of a solute across the membrane, usually against an electrochemical gradient. Active transport is tightly coupled to a source of metabolic energy, such as ATP hydrolysis or an electrochemically favorable ion gradient. Passive transport involves the movement of a solute down its electrochemical gradient. Transport proteins can be further classified as either carrier proteins or channel proteins. Carrier proteins, which can function in active or passive transport, bind to a specific solute to be transported and undergo a conformational change which transfers the bound solute across the membrane. Channel proteins, which only function in passive transport, form hydrophilic pores across the membrane. When the pores open, specific solutes, such as inorganic ions, pass through the membrane and down the electrochemical gradient of the solute.

Carrier proteins which transport a single solute from one side of the membrane to the other are called uniporters. In contrast, coupled transporters link the transfer of one solute with simultaneous or sequential transfer of a second solute, either in the same direction (symport) or in the opposite direction (antiport). For example, intestinal and kidney epithelium contains a variety of symporter systems driven by the sodium gradient that exists across the plasma membrane. Sodium moves into the cell down its electrochemical gradient and brings the solute into the cell with it. The sodium gradient that provides the driving force for solute uptake is maintained by the ubiquitous  $\text{Na}^+/\text{K}^+$  ATPase. Sodium-coupled transporters include the mammalian glucose transporter (SGLT1), iodide transporter (NIS), and multivitamin transporter (SMVT). All three transporters have twelve putative transmembrane segments, extracellular glycosylation sites, and cytoplasmically-oriented N- and C-termini. NIS plays a crucial role in the evaluation, diagnosis, and treatment of various thyroid pathologies because it is the molecular basis for radioiodide thyroid-imaging techniques and for specific targeting of radioisotopes to the thyroid gland (Levy, O. et al. (1997) Proc. Natl. Acad. Sci. USA 94:5568-5573). SMVT is expressed in the intestinal mucosa, kidney, and placenta, and is

implicated in the transport of the water-soluble vitamins, e.g., biotin and pantothenate (Prasad, P.D. et al. (1998) J. Biol. Chem. 273:7501-7506).

Transporters play a major role in the regulation of pH, excretion of drugs, and the cellular  $K^+/Na^+$  balance. Monocarboxylate anion transporters are proton-coupled symporters with a broad substrate specificity that includes L-lactate, pyruvate, and the ketone bodies acetate, acetoacetate, and beta-hydroxybutyrate. At least seven isoforms have been identified to date. The isoforms are predicted to have twelve transmembrane (TM) helical domains with a large intracellular loop between TM6 and TM7, and play a critical role in maintaining intracellular pH by removing the protons that are produced stoichiometrically with lactate during glycolysis. The best characterized H(+)-monocarboxylate transporter is that of the erythrocyte membrane, which transports L-lactate and a wide range of other aliphatic monocarboxylates. Other cells possess H(+)-linked monocarboxylate transporters with differing substrate and inhibitor selectivities. In particular, cardiac muscle and tumor cells have transporters that differ in their  $K_m$  values for certain substrates, including stereoselectivity for L- over D-lactate, and in their sensitivity to inhibitors. There are Na(+)-monocarboxylate cotransporters on the luminal surface of intestinal and kidney epithelia, which allow the uptake of lactate, pyruvate, and ketone bodies in these tissues. In addition, there are specific and selective transporters for organic cations and organic anions in organs including the kidney, intestine and liver. Organic anion transporters are selective for hydrophobic, charged molecules with electron-attracting side groups. Organic cation transporters, such as the ammonium transporter, mediate the secretion of a variety of drugs and endogenous metabolites, and contribute to the maintenance of intercellular pH. (Poole, R.C. and A.P. Halestrap (1993) Am. J. Physiol. 264:C761-C782; Price, N.T. et al. (1998) Biochem. J. 329:321-328; and Martinelle, K. and I. Haggstrom (1993) J. Biotechnol. 30: 339-350.)

The largest and most diverse family of transport proteins known is the ATP-binding cassette (ABC) transporters. As a family, ABC transporters can transport substances that differ markedly in chemical structure and size, ranging from small molecules such as ions, sugars, amino acids, peptides, and phospholipids, to lipopeptides, large proteins, and complex hydrophobic drugs. ABC proteins consist of four modules: two nucleotide-binding domains (NBD), which hydrolyze ATP to supply the energy required for transport, and two membrane-spanning domains (MSD), each containing six putative transmembrane segments. These four modules may be encoded by a single gene, as is the case for the cystic fibrosis transmembrane regulator (CFTR), or by separate genes. When encoded by separate genes, each gene product contains a single NBD and MSD. These "half-molecules" form homo- and heterodimers, such as Tap1 and Tap2, the endoplasmic reticulum-based major histocompatibility (MHC) peptide transport system. Several genetic diseases are attributed to defects in ABC transporters, such as the following diseases and their corresponding proteins: cystic fibrosis (CFTR, an ion channel), adrenoleukodystrophy (adrenoleukodystrophy protein, ALDP), Zellweger

syndrome (peroxisomal membrane protein-70, PMP70), and hyperinsulinemic hypoglycemia (sulfonylurea receptor, SUR). Overexpression of the multidrug resistance (MDR) protein, another ABC transporter, in human cancer cells makes the cells resistant to a variety of cytotoxic drugs used in chemotherapy (Taglicht, D. and S. Michaelis (1998) *Meth. Enzymol.* 292:131-163).

- 5 Transport of fatty acids across the plasma membrane can occur by diffusion, a high capacity, low affinity process. However, under normal physiological conditions a significant fraction of fatty acid transport appears to occur via a high affinity, low capacity protein-mediated transport process. Fatty acid transport protein (FATP), an integral membrane protein with four transmembrane segments, is expressed in tissues exhibiting high levels of plasma membrane fatty acid flux, such as muscle, heart,  
10 and adipose. Expression of FATP is upregulated in 3T3-L1 cells during adipose conversion, and expression in COS7 fibroblasts elevates uptake of long-chain fatty acids (Hui, T.Y. et al. (1998) *J. Biol. Chem.* 273:27420-27429).

### Ion Channels

- The electrical potential of a cell is generated and maintained by controlling the movement of  
15 ions across the plasma membrane. The movement of ions requires ion channels, which form an ion-selective pore within the membrane. There are two basic types of ion channels, ion transporters and gated ion channels. Ion transporters utilize the energy obtained from ATP hydrolysis to actively transport an ion against the ion's concentration gradient. Gated ion channels allow passive flow of an ion down the ion's electrochemical gradient under restricted conditions. Together, these types of ion  
20 channels generate, maintain, and utilize an electrochemical gradient that is used in 1) electrical impulse conduction down the axon of a nerve cell, 2) transport of molecules into cells against concentration gradients, 3) initiation of muscle contraction, and 4) endocrine cell secretion.

- Ion transporters generate and maintain the resting electrical potential of a cell. Utilizing the energy derived from ATP hydrolysis, they transport ions against the ion's concentration gradient.  
25 These transmembrane ATPases are divided into three families. The phosphorylated (P) class ion transporters, including  $\text{Na}^+\text{-K}^+$  ATPase,  $\text{Ca}^{2+}$ -ATPase, and  $\text{H}^+$ -ATPase, are activated by a phosphorylation event. P-class ion transporters are responsible for maintaining resting potential distributions such that cytosolic concentrations of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  are low and cytosolic concentration of  $\text{K}^+$  is high. The vacuolar (V) class of ion transporters includes  $\text{H}^+$  pumps on intracellular organelles,  
30 such as lysosomes and Golgi. V-class ion transporters are responsible for generating the low pH within the lumen of these organelles that is required for function. The coupling factor (F) class consists of  $\text{H}^+$  pumps in the mitochondria. F-class ion transporters utilize a proton gradient to generate ATP from ADP and inorganic phosphate ( $\text{P}_i$ ).

- The resting potential of the cell is utilized in many processes involving carrier proteins and  
35 gated ion channels. Carrier proteins utilize the resting potential to transport molecules into and out of



the cell. Amino acid and glucose transport into many cells is linked to sodium ion co-transport (symport) so that the movement of  $\text{Na}^+$  down an electrochemical gradient drives transport of the other molecule up a concentration gradient. Similarly, cardiac muscle links transfer of  $\text{Ca}^{2+}$  out of the cell with transport of  $\text{Na}^+$  into the cell (antiport).

5 Ion channels share common structural and mechanistic themes. The channel consists of four or five subunits or protein monomers that are arranged like a barrel in the plasma membrane. Each subunit typically consists of six potential transmembrane segments (S1, S2, S3, S4, S5, and S6). The center of the barrel forms a pore lined by  $\alpha$ -helices or  $\beta$ -strands. The side chains of the amino acid residues comprising the  $\alpha$ -helices or  $\beta$ -strands establish the charge (cation or anion) selectivity of the  
10 channel. The degree of selectivity, or what specific ions are allowed to pass through the channel, depends on the diameter of the narrowest part of the pore.

Gated ion channels control ion flow by regulating the opening and closing of pores. These channels are categorized according to the manner of regulating the gating function. Mechanically-gated channels open pores in response to mechanical stress, voltage-gated channels open pores in response to  
15 changes in membrane potential, and ligand-gated channels open pores in the presence of a specific ion, nucleotide, or neurotransmitter.

Voltage-gated  $\text{Na}^+$  and  $\text{K}^+$  channels are necessary for the function of electrically excitable cells, such as nerve and muscle cells. Action potentials, which lead to neurotransmitter release and muscle contraction, arise from large, transient changes in the permeability of the membrane to  $\text{Na}^+$  and  $\text{K}^+$  ions.  
20 Depolarization of the membrane beyond the threshold level opens voltage-gated  $\text{Na}^+$  channels. Sodium ions flow into the cell, further depolarizing the membrane and opening more voltage-gated  $\text{Na}^+$  channels, which propagates the depolarization down the length of the cell. Depolarization also opens voltage-gated potassium channels. Consequently, potassium ions flow outward, which leads to repolarization of the membrane. Voltage-gated channels utilize charged residues in the fourth  
25 transmembrane segment (S4) to sense voltage change. The open state lasts only about 1 millisecond, at which time the channel spontaneously converts into an inactive state that cannot be opened irrespective of the membrane potential. Inactivation is mediated by the channel's N-terminus, which acts as a plug that closes the pore. The transition from an inactive to a closed state requires a return to resting potential.

30 Voltage-gated  $\text{Na}^+$  channels are heterotrimeric complexes composed of a 260 kDa pore forming  $\alpha$  subunit that associates with two smaller auxiliary subunits,  $\beta 1$  and  $\beta 2$ . The  $\beta 2$  subunit is an integral membrane glycoprotein that contains an extracellular Ig domain, and its association with  $\alpha$  and  $\beta 1$  subunits correlates with increased functional expression of the channel, a change in its gating properties, and an increase in whole cell capacitance due to an increase in membrane surface area.  
35 (Isom, L.L. et al. (1995) Cell 83:433-442.)

Voltage-gated  $\text{Ca}^{2+}$  channels are involved in presynaptic neurotransmitter release, and heart and skeletal muscle contraction. The voltage-gated  $\text{Ca}^{2+}$  channels from skeletal muscle (L-type) and brain (N-type) have been purified, and though their functions differ dramatically, they have similar subunit compositions. The channels are composed of three subunits. The  $\alpha_1$  subunit forms the membrane pore and voltage sensor, while the  $\alpha_2\delta$  and  $\beta$  subunits modulate the voltage-dependence, gating properties, and the current amplitude of the channel. These subunits are encoded by at least six  $\alpha_1$ , one  $\alpha_2\delta$ , and four  $\beta$  genes. A fourth subunit,  $\gamma$ , has been identified in skeletal muscle. (Walker, D. et al. (1998) *J. Biol. Chem.* 273:2361-2367; and Jay, S.D. et al. (1990) *Science* 248:490-492.)

Chloride channels are necessary in endocrine secretion and in regulation of cytosolic and organelle pH. In secretory epithelial cells,  $\text{Cl}^-$  enters the cell across a basolateral membrane through an  $\text{Na}^+$ ,  $\text{K}^+/\text{Cl}^-$  cotransporter, accumulating in the cell above its electrochemical equilibrium concentration. Secretion of  $\text{Cl}^-$  from the apical surface, in response to hormonal stimulation, leads to flow of  $\text{Na}^+$  and water into the secretory lumen. The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel encoded by the gene for cystic fibrosis, a common fatal genetic disorder in humans. Loss of CFTR function decreases transepithelial water secretion and, as a result, the layers of mucus that coat the respiratory tree, pancreatic ducts, and intestine are dehydrated and difficult to clear. The resulting blockage of these sites leads to pancreatic insufficiency, "meconium ileus", and devastating "chronic obstructive pulmonary disease" (Al-Awqati, Q. et al. (1992) *J. Exp. Biol.* 172:245-266).

Many intracellular organelles contain  $\text{H}^+$ -ATPase pumps that generate transmembrane pH and electrochemical differences by moving protons from the cytosol to the organelle lumen. If the membrane of the organelle is permeable to other ions, then the electrochemical gradient can be abrogated without affecting the pH differential. In fact, removal of the electrochemical barrier allows more  $\text{H}^+$  to be pumped across the membrane, increasing the pH differential.  $\text{Cl}^-$  is the sole counterion of  $\text{H}^+$  translocation in a number of organelles, including chromaffin granules, Golgi vesicles, lysosomes, and endosomes. Functions that require a low vacuolar pH include uptake of small molecules such as biogenic amines in chromaffin granules, processing of vacuolar constituents such as pro-hormones by proteolytic enzymes, and protein degradation in lysosomes (Al-Awqati, *supra*).

Ligand-gated channels open their pores when an extracellular or intracellular mediator binds to the channel. Neurotransmitter-gated channels are channels that open when a neurotransmitter binds to their extracellular domain. These channels exist in the postsynaptic membrane of nerve or muscle cells. There are two types of neurotransmitter-gated channels. Sodium channels open in response to excitatory neurotransmitters, such as acetylcholine, glutamate, and serotonin. This opening causes an influx of  $\text{Na}^+$  and produces the initial localized depolarization that activates the voltage-gated channels and starts the action potential. Chloride channels open in response to inhibitory neurotransmitters, such as  $\gamma$ -aminobutyric acid (GABA) and glycine, leading to hyperpolarization of the membrane and the

subsequent generation of an action potential.

Ligand-gated channels can be regulated by intracellular second messengers. Calcium-activated  $K^+$  channels are gated by internal calcium ions. In nerve cells, an influx of calcium during depolarization opens  $K^+$  channels to modulate the magnitude of the action potential (Ishi, T.M. et al. (1997) Proc. Natl. Acad. Sci. USA 94:11651-11656). Cyclic nucleotide-gated (CNG) channels are gated by cytosolic cyclic nucleotides. The best examples of these are the cAMP-gated  $Na^+$  channels involved in olfaction and the cGMP-gated cation channels involved in vision. Both systems involve ligand-mediated activation of a G-protein coupled receptor which then alters the level of cyclic nucleotide within the cell.

Ion channels are expressed in a number of tissues where they are implicated in a variety of processes. CNG channels, while abundantly expressed in photoreceptor and olfactory sensory cells, are also found in kidney, lung, pineal, retinal ganglion cells, testis, aorta, and brain. Calcium-activated  $K^+$  channels may be responsible for the vasodilatory effects of bradykinin in the kidney and for shunting excess  $K^+$  from brain capillary endothelial cells into the blood. They are also implicated in repolarizing granulocytes after agonist-stimulated depolarization (Ishi, supra). Ion channels have been the target for many drug therapies. Neurotransmitter-gated channels have been targeted in therapies for treatment of insomnia, anxiety, depression, and schizophrenia. Voltage-gated channels have been targeted in therapies for arrhythmia, ischemic stroke, head trauma, and neurodegenerative disease (Taylor, C.P. and L.S. Narasimhan (1997) Adv. Pharmacol. 39:47-98).

#### Disease Correlation

The etiology of numerous human diseases and disorders can be attributed to defects in the transport of molecules across membranes. Defects in the trafficking of membrane-bound transporters and ion channels are associated with several disorders, e.g. cystic fibrosis, glucose-galactose malabsorption syndrome, hypercholesterolemia, von Gierke disease, and certain forms of diabetes mellitus. Single-gene defect diseases resulting in an inability to transport small molecules across membranes include, e.g., cystinuria, iminoglycinuria, Hartup disease, and Fanconi disease (van't Hoff, W.G. (1996) Exp. Nephrol. 4:253-262; Talente, G.M. et al. (1994) Ann. Intern. Med. 120:218-226; and Chillon, M. et al. (1995) New Engl. J. Med. 332:1475-1480).

#### Protein Modification and Maintenance Molecules

The cellular processes regulating modification and maintenance of protein molecules coordinate their conformation, stabilization, and degradation. Each of these processes is mediated by key enzymes or proteins such as proteases, protease inhibitors, transferases, isomerases, and molecular chaperones.

#### Proteases

Proteases cleave proteins and peptides at the peptide bond that forms the backbone of the peptide and protein chain. Proteolytic processing is essential to cell growth, differentiation, remodeling, and homeostasis as well as inflammation and immune response. Typical protein half-lives range from hours to a few days, so that within all living cells, precursor proteins are being  
 5 cleaved to their active form, signal sequences proteolytically removed from targeted proteins, and aged or defective proteins degraded by proteolysis. Proteases function in bacterial, parasitic, and viral invasion and replication within a host. Four principal categories of mammalian proteases have been identified based on active site structure, mechanism of action, and overall three-dimensional structure. (Beynon, R.J. and J.S. Bond (1994) Proteolytic Enzymes: A Practical Approach, Oxford University  
 10 Press, New York NY, pp. 1-5).

The serine proteases (SPs) have a serine residue, usually within a conserved sequence, in an active site composed of the serine, an aspartate, and a histidine residue. SPs include the digestive enzymes trypsin and chymotrypsin, components of the complement cascade and the blood-clotting cascade, and enzymes that control extracellular protein degradation. The main SP sub-families are  
 15 trypsinases, which cleave after arginine or lysine; aspartases, which cleave after aspartate; chymases, which cleave after phenylalanine or leucine; metases, which cleavage after methionine; and serases which cleave after serine. Enterokinase, the initiator of intestinal digestion, is a serine protease found in the intestinal brush border, where it cleaves the acidic propeptide from trypsinogen to yield active trypsin (Kitamoto, Y. et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:7588-7592).  
 20 Prolylcarboxypeptidase, a lysosomal serine peptidase that cleaves peptides such as angiotensin II and III and [des-Arg9] bradykinin, shares sequence homology with members of both the serine carboxypeptidase and prolylendopeptidase families (Tan, F. et al. (1993) *J. Biol. Chem.* 268:16631-16638).

Cysteine proteases (CPs) have a cysteine as the major catalytic residue at an active site where  
 25 catalysis proceeds via an intermediate thiol ester and is facilitated by adjacent histidine and aspartic acid residues. CPs are involved in diverse cellular processes ranging from the processing of precursor proteins to intracellular degradation. Mammalian CPs include lysosomal cathepsins and cytosolic calcium activated proteases, calpains. CPs are produced by monocytes, macrophages and other cells of the immune system which migrate to sites of inflammation and secrete molecules involved in  
 30 tissue repair. Overabundance of these repair molecules plays a role in certain disorders. In autoimmune diseases such as rheumatoid arthritis, secretion of the cysteine peptidase cathepsin C degrades collagen, laminin, elastin and other structural proteins found in the extracellular matrix of bones.

Aspartic proteases are members of the cathepsin family of lysosomal proteases and include  
 35 pepsin A, gastricsin, chymosin, renin, and cathepsins D and E. Aspartic proteases have a pair of

aspartic acid residues in the active site, and are most active in the pH 2 - 3 range, in which one of the aspartate residues is ionized, the other un-ionized. Aspartic proteases include bacterial penicillopepsin, mammalian pepsin, renin, chymosin, and certain fungal proteases. Abnormal regulation and expression of cathepsins is evident in various inflammatory disease states. In cells isolated from inflamed synovia, the mRNA for stromelysin, cytokines, TIMP-1, cathepsin, gelatinase, and other molecules is preferentially expressed. Expression of cathepsins L and D is elevated in synovial tissues from patients with rheumatoid arthritis and osteoarthritis. Cathepsin L expression may also contribute to the influx of mononuclear cells which exacerbates the destruction of the rheumatoid synovium. (Keyszer, G.M. (1995) *Arthritis Rheum.* 38:976-984.) The increased expression and differential regulation of the cathepsins are linked to the metastatic potential of a variety of cancers and as such are of therapeutic and prognostic interest (Chambers, A.F. et al. (1993) *Crit. Rev. Oncog.* 4:95-114).

Metalloproteases have active sites that include two glutamic acid residues and one histidine residue that serve as binding sites for zinc. Carboxypeptidases A and B are the principal mammalian metalloproteases. Both are exoproteases of similar structure and active sites. Carboxypeptidase A, like chymotrypsin, prefers C-terminal aromatic and aliphatic side chains of hydrophobic nature, whereas carboxypeptidase B is directed toward basic arginine and lysine residues. Glycoprotease (GCP), or O-sialoglycoprotein endopeptidase, is a metalloprotease which specifically cleaves O-sialoglycoproteins such as glycophorin A. Another metalloprotease, placental leucine aminopeptidase (P-LAP) degrades several peptide hormones such as oxytocin and vasopressin, suggesting a role in maintaining homeostasis during pregnancy, and is expressed in several tissues (Rogi, T. et al. (1996) *J. Biol. Chem.* 271:56-61).

Ubiquitin proteases are associated with the ubiquitin conjugation system (UCS), a major pathway for the degradation of cellular proteins in eukaryotic cells and some bacteria. The UCS mediates the elimination of abnormal proteins and regulates the half-lives of important regulatory proteins that control cellular processes such as gene transcription and cell cycle progression. In the UCS pathway, proteins targeted for degradation are conjugated to a ubiquitin, a small heat stable protein. The ubiquitinated protein is then recognized and degraded by proteasome, a large, multisubunit proteolytic enzyme complex, and ubiquitin is released for reutilization by ubiquitin protease. The UCS is implicated in the degradation of mitotic cyclic kinases, oncoproteins, tumor suppressor genes such as p53, viral proteins, cell surface receptors associated with signal transduction, transcriptional regulators, and mutated or damaged proteins (Ciechanover, A. (1994) *Cell* 79:13-21). A murine proto-oncogene, *Unp*, encodes a nuclear ubiquitin protease whose overexpression leads to oncogenic transformation of NIH3T3 cells, and the human homolog of this gene is consistently elevated in small cell tumors and adenocarcinomas of the lung (Gray, D.A.

(1995) Oncogene 10:2179-2183).

### Signal Peptidases

The mechanism for the translocation process into the endoplasmic reticulum (ER) involves the recognition of an N-terminal signal peptide on the elongating protein. The signal peptide directs the protein and attached ribosome to a receptor on the ER membrane. The polypeptide chain passes through a pore in the ER membrane into the lumen while the N-terminal signal peptide remains attached at the membrane surface. The process is completed when signal peptidase located inside the ER cleaves the signal peptide from the protein and releases the protein into the lumen.

### Protease Inhibitors

Protease inhibitors and other regulators of protease activity control the activity and effects of proteases. Protease inhibitors have been shown to control pathogenesis in animal models of proteolytic disorders (Murphy, G. (1991) Agents Actions Suppl. 35:69-76). Low levels of the cystatins, low molecular weight inhibitors of the cysteine proteases, correlate with malignant progression of tumors. (Calkins, C. et al (1995) Biol. Biochem. Hoppe Seyler 376:71-80). Serpins are inhibitors of mammalian plasma serine proteases. Many serpins serve to regulate the blood clotting cascade and/or the complement cascade in mammals. Sp32 is a positive regulator of the mammalian acrosomal protease, acrosin, that binds the proenzyme, proacrosin, and thereby aides in packaging the enzyme into the acrosomal matrix (Baba, T. et al. (1994) J. Biol. Chem. 269:10133-10140). The Kunitz family of serine protease inhibitors are characterized by one or more "Kunitz domains" containing a series of cysteine residues that are regularly spaced over approximately 50 amino acid residues and form three intrachain disulfide bonds. Members of this family include aprotinin, tissue factor pathway inhibitor (TFPI-1 and TFPI-2), inter- $\alpha$ -trypsin inhibitor, and bikunin. (Marlor, C.W. et al. (1997) J. Biol. Chem. 272:12202-12208.) Members of this family are potent inhibitors (in the nanomolar range) against serine proteases such as kallikrein and plasmin. Aprotinin has clinical utility in reduction of perioperative blood loss.

A major portion of all proteins synthesized in eukaryotic cells are synthesized on the cytosolic surface of the endoplasmic reticulum (ER). Before these immature proteins are distributed to other organelles in the cell or are secreted, they must be transported into the interior lumen of the ER where post-translational modifications are performed. These modifications include protein folding and the formation of disulfide bonds, and N-linked glycosylations.

### Protein Isomerases

Protein folding in the ER is aided by two principal types of protein isomerases, protein disulfide isomerase (PDI), and peptidyl-prolyl isomerase (PPI). PDI catalyzes the oxidation of free sulfhydryl groups in cysteine residues to form intramolecular disulfide bonds in proteins. PPI, an enzyme that catalyzes the isomerization of certain proline imidic bonds in oligopeptides and proteins,

is considered to govern one of the rate limiting steps in the folding of many proteins to their final functional conformation. The cyclophilins represent a major class of PPI that was originally identified as the major receptor for the immunosuppressive drug cyclosporin A (Handschumacher, R.E. et al. (1984) Science 226: 544-547).

## 5 Protein Glycosylation

The glycosylation of most soluble secreted and membrane-bound proteins by oligosaccharides linked to asparagine residues in proteins is also performed in the ER. This reaction is catalyzed by a membrane-bound enzyme, oligosaccharyl transferase. Although the exact purpose of this "N-linked" glycosylation is unknown, the presence of oligosaccharides tends to make a glycoprotein resistant to protease digestion. In addition, oligosaccharides attached to cell-surface proteins called selectins are known to function in cell-cell adhesion processes (Alberts, B. et al. (1994) Molecular Biology of the Cell, Garland Publishing Co., New York NY, p.608). "O-linked" glycosylation of proteins also occurs in the ER by the addition of N-acetylgalactosamine to the hydroxyl group of a serine or threonine residue followed by the sequential addition of other sugar residues to the first. This process is catalysed by a series of glycosyltransferases each specific for a particular donor sugar nucleotide and acceptor molecule (Lodish, H. et al. (1995) Molecular Cell Biology, W.H. Freeman and Co., New York NY, pp.700-708). In many cases, both N- and O-linked oligosaccharides appear to be required for the secretion of proteins or the movement of plasma membrane glycoproteins to the cell surface.

An additional glycosylation mechanism operates in the ER specifically to target lysosomal enzymes to lysosomes and prevent their secretion. Lysosomal enzymes in the ER receive an N-linked oligosaccharide, like plasma membrane and secreted proteins, but are then phosphorylated on one or two mannose residues. The phosphorylation of mannose residues occurs in two steps, the first step being the addition of an N-acetylglucosamine phosphate residue by N-acetylglucosamine phosphotransferase, and the second the removal of the N-acetylglucosamine group by phosphodiesterase. The phosphorylated mannose residue then targets the lysosomal enzyme to a mannose 6-phosphate receptor which transports it to a lysosome vesicle (Lodish, supra, pp. 708-711).

## Chaperones

Molecular chaperones are proteins that aid in the proper folding of immature proteins and refolding of improperly folded ones, the assembly of protein subunits, and in the transport of unfolded proteins across membranes. Chaperones are also called heat-shock proteins (hsp) because of their tendency to be expressed in dramatically increased amounts following brief exposure of cells to elevated temperatures. This latter property most likely reflects their need in the refolding of proteins that have become denatured by the high temperatures. Chaperones may be divided into several classes according to their location, function, and molecular weight, and include hsp60, TCP1, hsp70,

hsp40 (also called DnaJ), and hsp90. For example, hsp90 binds to steroid hormone receptors, represses transcription in the absence of the ligand, and provides proper folding of the ligand-binding domain of the receptor in the presence of the hormone (Burston, S.G. and A.R. Clarke (1995) *Essays Biochem.* 29:125-136). Hsp60 and hsp70 chaperones aid in the transport and folding of newly synthesized proteins. Hsp70 acts early in protein folding, binding a newly synthesized protein before it leaves the ribosome and transporting the protein to the mitochondria or ER before releasing the folded protein. Hsp60, along with hsp10, binds misfolded proteins and gives them the opportunity to refold correctly. All chaperones share an affinity for hydrophobic patches on incompletely folded proteins and the ability to hydrolyze ATP. The energy of ATP hydrolysis is used to release the hsp-bound protein in its properly folded state (Alberts, supra, pp 214, 571-572).

#### Nucleic Acid Synthesis and Modification Molecules

SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, and SEQ ID NO:20 encode, for example, nucleic acid synthesis and modification molecules.

#### 15 Polymerases

DNA and RNA replication are critical processes for cell replication and function. DNA and RNA replication are mediated by the enzymes DNA and RNA polymerase, respectively, by a "templating" process in which the nucleotide sequence of a DNA or RNA strand is copied by complementary base-pairing into a complementary nucleic acid sequence of either DNA or RNA. However, there are fundamental differences between the two processes.

DNA polymerase catalyzes the stepwise addition of a deoxyribonucleotide to the 3'-OH end of a polynucleotide strand (the primer strand) that is paired to a second (template) strand. The new DNA strand therefore grows in the 5' to 3' direction (Alberts, B. et al. (1994) The Molecular Biology of the Cell, Garland Publishing Inc., New York NY, pp. 251-254). The substrates for the polymerization reaction are the corresponding deoxynucleotide triphosphates which must base-pair with the correct nucleotide on the template strand in order to be recognized by the polymerase. Because DNA exists as a double-stranded helix, each of the two strands may serve as a template for the formation of a new complementary strand. Each of the two daughter cells of the dividing cell therefore inherits a new DNA double helix containing one old and one new strand. Thus, DNA is said to be replicated "semiconservatively" by DNA polymerase. In addition to the synthesis of new DNA, DNA polymerase is also involved in the repair of damaged DNA as discussed below under "Ligases."

In contrast to DNA polymerase, RNA polymerase uses a DNA template strand to "transcribe" DNA into RNA using ribonucleotide triphosphates as substrates. Like DNA polymerization, RNA polymerization proceeds in a 5' to 3' direction by addition of a ribonucleoside monophosphate to the 3'-OH end of a growing RNA chain. DNA transcription generates messenger RNAs (mRNA) that carry



information for protein synthesis, as well as the transfer, ribosomal, and other RNAs that have structural or catalytic functions. In eukaryotes, three discrete RNA polymerases synthesize the three different types of RNA (Alberts, supra, pp. 367-368). RNA polymerase I makes the large ribosomal RNAs, RNA polymerase II makes the mRNAs that will be translated into proteins, and RNA polymerase III makes a variety of small, stable RNAs, including 5S ribosomal RNA and the transfer RNAs (tRNA). In all cases, RNA synthesis is initiated by binding of the RNA polymerase to a promoter region on the DNA and synthesis begins at a start site within the promoter. Synthesis is completed at a broad, general stop or termination region in the DNA where both the polymerase and the completed RNA chain are released.

#### Ligases

DNA repair is the process by which accidental base changes, such as those produced by oxidative damage, hydrolytic attack, or uncontrolled methylation of DNA are corrected before replication or transcription of the DNA can occur. Because of the efficiency of the DNA repair process, fewer than one in one thousand accidental base changes causes a mutation (Alberts, supra, pp. 245-249). The three steps common to most types of DNA repair are (1) excision of the damaged or altered base or nucleotide by DNA nucleases, leaving a gap; (2) insertion of the correct nucleotide in this gap by DNA polymerase using the complementary strand as the template; and (3) sealing the break left between the inserted nucleotide(s) and the existing DNA strand by DNA ligase. In the last reaction, DNA ligase uses the energy from ATP hydrolysis to activate the 5' end of the broken phosphodiester bond before forming the new bond with the 3'-OH of the DNA strand. In Bloom's syndrome, an inherited human disease, individuals are partially deficient in DNA ligation and consequently have an increased incidence of cancer (Alberts, supra, p. 247).

#### Nucleases

Nucleases comprise both enzymes that hydrolyze DNA (DNase) and RNA (RNase). They serve different purposes in nucleic acid metabolism. Nucleases hydrolyze the phosphodiester bonds between adjacent nucleotides either at internal positions (endonucleases) or at the terminal 3' or 5' nucleotide positions (exonucleases). A DNA exonuclease activity in DNA polymerase, for example, serves to remove improperly paired nucleotides attached to the 3'-OH end of the growing DNA strand by the polymerase and thereby serves a "proofreading" function. As mentioned above, DNA endonuclease activity is involved in the excision step of the DNA repair process.

RNases also serve a variety of functions. For example, RNase P is a ribonucleoprotein enzyme which cleaves the 5' end of pre-tRNAs as part of their maturation process. RNase H digests the RNA strand of an RNA/DNA hybrid. Such hybrids occur in cells invaded by retroviruses, and RNase H is an important enzyme in the retroviral replication cycle. Pancreatic RNase secreted by the pancreas into the intestine hydrolyzes RNA present in ingested foods. RNase activity in serum and cell extracts is

elevated in a variety of cancers and infectious diseases (Schein, C.H. (1997) Nat. Biotechnol. 15:529-536). Regulation of RNase activity is being investigated as a means to control tumor angiogenesis, allergic reactions, viral infection and replication, and fungal infections.

#### Methylases

5 Methylation of specific nucleotides occurs in both DNA and RNA, and serves different functions in the two macromolecules. Methylation of cytosine residues to form 5-methyl cytosine in DNA occurs specifically at CG sequences which are base-paired with one another in the DNA double-helix. This pattern of methylation is passed from generation to generation during DNA replication by an enzyme called "maintenance methylase" that acts preferentially on those CG sequences that are base-  
10 paired with a CG sequence that is already methylated. Such methylation appears to distinguish active from inactive genes by preventing the binding of regulatory proteins that "turn on" the gene, but permit the binding of proteins that inactivate the gene (Alberts, supra, pp. 448-451). In RNA metabolism, "tRNA methylase" produces one of several nucleotide modifications in tRNA that affect the conformation and base-pairing of the molecule and facilitate the recognition of the appropriate mRNA  
15 codons by specific tRNAs. The primary methylation pattern is the dimethylation of guanine residues to form N,N-dimethyl guanine.

#### Helicases and Single-Stranded Binding Proteins

Helicases are enzymes that destabilize and unwind double helix structures in both DNA and RNA. Since DNA replication occurs more or less simultaneously on both strands, the two strands must  
20 first separate to generate a replication "fork" for DNA polymerase to act on. Two types of replication proteins contribute to this process, DNA helicases and single-stranded binding proteins. DNA helicases hydrolyze ATP and use the energy of hydrolysis to separate the DNA strands. Single-stranded binding proteins (SSBs) then bind to the exposed DNA strands without covering the bases, thereby temporarily stabilizing them for templating by the DNA polymerase (Alberts, supra, pp. 255-256).

25 RNA helicases also alter and regulate RNA conformation and secondary structure. Like the DNA helicases, RNA helicases utilize energy derived from ATP hydrolysis to destabilize and unwind RNA duplexes. The most well-characterized and ubiquitous family of RNA helicases is the DEAD-box family, so named for the conserved B-type ATP-binding motif which is diagnostic of proteins in this family. Over 40 DEAD-box helicases have been identified in organisms as diverse as bacteria, insects,  
30 yeast, amphibians, mammals, and plants. DEAD-box helicases function in diverse processes such as translation initiation, splicing, ribosome assembly, and RNA editing, transport, and stability. Some DEAD-box helicases play tissue- and stage-specific roles in spermatogenesis and embryogenesis. Overexpression of the DEAD-box 1 protein (DDX1) may play a role in the progression of neuroblastoma (Nb) and retinoblastoma (Rb) tumors (Godbout, R. et al. (1998) J. Biol. Chem.  
35 273:21161-21168). These observations suggest that DDX1 may promote or enhance tumor

progression by altering the normal secondary structure and expression levels of RNA in cancer cells. Other DEAD-box helicases have been implicated either directly or indirectly in tumorigenesis (Discussed in Godbout, supra). For example, murine p68 is mutated in ultraviolet light-induced tumors, and human DDX6 is located at a chromosomal breakpoint associated with B-cell lymphoma.

- 5 Similarly, a chimeric protein comprised of DDX10 and NUP98, a nucleoporin protein, may be involved in the pathogenesis of certain myeloid malignancies.

#### Topoisomerases

- Besides the need to separate DNA strands prior to replication, the two strands must be “unwound” from one another prior to their separation by DNA helicases. This function is performed by proteins known as DNA topoisomerases. DNA topoisomerase effectively acts as a reversible nuclease that hydrolyzes a phosphodiesterase bond in a DNA strand, permitting the two strands to rotate freely about one another to remove the strain of the helix, and then rejoins the original phosphodiester bond between the two strands. Two types of DNA topoisomerase exist, types I and II. DNA Topoisomerase I causes a single-strand break in a DNA helix to allow the rotation of the two strands of the helix about the remaining phosphodiester bond in the opposite strand. DNA topoisomerase II causes a transient break in both strands of a DNA helix where two double helices cross over one another. This type of topoisomerase can efficiently separate two interlocked DNA circles (Alberts, supra, pp.260-262). Type II topoisomerases are largely confined to proliferating cells in eukaryotes, such as cancer cells. For this reason they are targets for anticancer drugs. Topoisomerase II has been implicated in multi-drug resistance (MDR) as it appears to aid in the repair of DNA damage inflicted by DNA binding agents such as doxorubicin and vincristine.
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#### Recombinases

- Genetic recombination is the process of rearranging DNA sequences within an organism's genome to provide genetic variation for the organism in response to changes in the environment. DNA recombination allows variation in the particular combination of genes present in an individual's genome, as well as the timing and level of expression of these genes (see Alberts, supra, pp. 263-273). Two broad classes of genetic recombination are commonly recognized, general recombination and site-specific recombination. General recombination involves genetic exchange between any homologous pair of DNA sequences usually located on two copies of the same chromosome. The process is aided by enzymes called recombinases that “nick” one strand of a DNA duplex more or less randomly and permit exchange with the complementary strand of another duplex. The process does not normally change the arrangement of genes on a chromosome. In site-specific recombination, the recombinase recognizes specific nucleotide sequences present in one or both of the recombining molecules. Base-pairing is not involved in this form of recombination and therefore does not require DNA homology between the recombining molecules. Unlike general recombination, this form of recombination can
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alter the relative positions of nucleotide sequences in chromosomes.

#### Splicing Factors

- Various proteins are necessary for processing of transcribed RNAs in the nucleus. Pre-mRNA processing steps include capping at the 5' end with methylguanosine, polyadenylating the 3' end, and
- 5 splicing to remove introns. The primary RNA transcript from DNA is a faithful copy of the gene containing both exon and intron sequences, and the latter sequences must be cut out of the RNA transcript to produce an mRNA that codes for a protein. This "splicing" of the mRNA sequence takes place in the nucleus with the aid of a large, multicomponent ribonucleoprotein complex known as a spliceosome. The spliceosomal complex is composed of five small nuclear ribonucleoprotein particles
- 10 (snRNPs) designated U1, U2, U4, U5, and U6, and a number of additional proteins. Each snRNP contains a single species of snRNA and about ten proteins. The RNA components of some snRNPs recognize and base pair with intron consensus sequences. The protein components mediate spliceosome assembly and the splicing reaction. Autoantibodies to snRNP proteins are found in the blood of patients with systemic lupus erythematosus (Stryer, L. (1995) Biochemistry, W.H. Freeman and
- 15 Company, New York NY, p. 863).

#### Adhesion Molecules

SEQ ID NO:21 and SEQ ID NO:22 encode, for example, adhesion molecules.

- The surface of a cell is rich in transmembrane proteoglycans, glycoproteins, glycolipids, and
- 20 receptors. These macromolecules mediate adhesion with other cells and with components of the extracellular matrix (ECM). The interaction of the cell with its surroundings profoundly influences cell shape, strength, flexibility, motility, and adhesion. These dynamic properties are intimately associated with signal transduction pathways controlling cell proliferation and differentiation, tissue construction, and embryonic development.

#### Cadherins

- Cadherins comprise a family of calcium-dependent glycoproteins that function in mediating cell-cell adhesion in virtually all solid tissues of multicellular organisms. These proteins share multiple repeats of a cadherin-specific motif, and the repeats form the folding units of the cadherin extracellular domain. Cadherin molecules cooperate to form focal contacts, or adhesion plaques, between adjacent
- 30 epithelial cells. The cadherin family includes the classical cadherins and protocadherins. Classical cadherins include the E-cadherin, N-cadherin, and P-cadherin subfamilies. E-cadherin is present on many types of epithelial cells and is especially important for embryonic development. N-cadherin is present on nerve, muscle, and lens cells and is also critical for embryonic development. P-cadherin is present on cells of the placenta and epidermis. Recent studies report that protocadherins are involved in
- 35 a variety of cell-cell interactions (Suzuki, S.T. (1996) *J. Cell Sci.* 109:2609-2611). The intracellular

anchorage of cadherins is regulated by their dynamic association with catenins, a family of cytoplasmic signal transduction proteins associated with the actin cytoskeleton. The anchorage of cadherins to the actin cytoskeleton appears to be regulated by protein tyrosine phosphorylation, and the cadherins are the target of phosphorylation-induced junctional disassembly (Aberle, H. et al. (1996) J. Cell. Biochem. 61:514-523).

#### Integrins

Integrins are ubiquitous transmembrane adhesion molecules that link the ECM to the internal cytoskeleton. Integrins are composed of two noncovalently associated transmembrane glycoprotein subunits called  $\alpha$  and  $\beta$ . Integrins function as receptors that play a role in signal transduction. For example, binding of integrin to its extracellular ligand may stimulate changes in intracellular calcium levels or protein kinase activity (Sjaastad, M.D. and W.J. Nelson (1997) BioEssays 19:47-55). At least ten cell surface receptors of the integrin family recognize the ECM component fibronectin, which is involved in many different biological processes including cell migration and embryogenesis (Johansson, S. et al. (1997) Front. Biosci. 2:D126-D146).

#### Lectins

Lectins comprise a ubiquitous family of extracellular glycoproteins which bind cell surface carbohydrates specifically and reversibly, resulting in the agglutination of cells (reviewed in Drickamer, K. and M.E. Taylor (1993) Annu. Rev. Cell Biol. 9:237-264). This function is particularly important for activation of the immune response. Lectins mediate the agglutination and mitogenic stimulation of lymphocytes at sites of inflammation (Lasky, L.A. (1991) J. Cell. Biochem. 45:139-146; Paietta, E. et al. (1989) J. Immunol. 143:2850-2857).

Lectins are further classified into subfamilies based on carbohydrate-binding specificity and other criteria. The galectin subfamily, in particular, includes lectins that bind  $\beta$ -galactoside carbohydrate moieties in a thiol-dependent manner (reviewed in Hadari, Y.R. et al. (1998) J. Biol. Chem. 270:3447-3453). Galectins are widely expressed and developmentally regulated. Because all galectins lack an N-terminal signal peptide, it is suggested that galectins are externalized through an atypical secretory mechanism. Two classes of galectins have been defined based on molecular weight and oligomerization properties. Small galectins form homodimers and are about 14 to 16 kilodaltons in mass, while large galectins are monomeric and about 29-37 kilodaltons.

Galectins contain a characteristic carbohydrate recognition domain (CRD). The CRD is about 140 amino acids and contains several stretches of about 1 - 10 amino acids which are highly conserved among all galectins. A particular 6-amino acid motif within the CRD contains conserved tryptophan and arginine residues which are critical for carbohydrate binding. The CRD of some galectins also contains cysteine residues which may be important for disulfide bond formation. Secondary structure predictions indicate that the CRD forms several  $\beta$ -sheets.

Galectins play a number of roles in diseases and conditions associated with cell-cell and cell-matrix interactions. For example, certain galectins associate with sites of inflammation and bind to cell surface immunoglobulin E molecules. In addition, galectins may play an important role in cancer metastasis. Galectin overexpression is correlated with the metastatic potential of cancers in humans and mice. Moreover, anti-galectin antibodies inhibit processes associated with cell transformation, such as cell aggregation and anchorage-independent growth (See, for example, Su, Z.-Z. et al. (1996) Proc. Natl. Acad. Sci. USA 93:7252-7257).

### Selectins

Selectins, or LEC-CAMs, comprise a specialized lectin subfamily involved primarily in inflammation and leukocyte adhesion (Reviewed in Lasky, supra). Selectins mediate the recruitment of leukocytes from the circulation to sites of acute inflammation and are expressed on the surface of vascular endothelial cells in response to cytokine signaling. Selectins bind to specific ligands on the leukocyte cell membrane and enable the leukocyte to adhere to and migrate along the endothelial surface. Binding of selectin to its ligand leads to polarized rearrangement of the actin cytoskeleton and stimulates signal transduction within the leukocyte (Brenner, B. et al. (1997) Biochem. Biophys. Res. Commun. 231:802-807; Hidari, K.I. et al. (1997) J. Biol. Chem. 272:28750-28756). Members of the selectin family possess three characteristic motifs: a lectin or carbohydrate recognition domain; an epidermal growth factor-like domain; and a variable number of short consensus repeats (scr or "sushi" repeats) which are also present in complement regulatory proteins. The selectins include lymphocyte adhesion molecule-1 (Lam-1 or L-selectin), endothelial leukocyte adhesion molecule-1 (ELAM-1 or E-selectin), and granule membrane protein-140 (GMP-140 or P-selectin) (Johnston, G.I. et al. (1989) Cell 56:1033-1044).

### Antigen Recognition Molecules

All vertebrates have developed sophisticated and complex immune systems that provide protection from viral, bacterial, fungal, and parasitic infections. A key feature of the immune system is its ability to distinguish foreign molecules, or antigens, from "self" molecules. This ability is mediated primarily by secreted and transmembrane proteins expressed by leukocytes (white blood cells) such as lymphocytes, granulocytes, and monocytes. Most of these proteins belong to the immunoglobulin (Ig) superfamily, members of which contain one or more repeats of a conserved structural domain. This Ig domain is comprised of antiparallel  $\beta$  sheets joined by a disulfide bond in an arrangement called the Ig fold. Members of the Ig superfamily include T-cell receptors, major histocompatibility (MHC) proteins, antibodies, and immune cell-specific surface markers such as CD4, CD8, and CD28.

MHC proteins are cell surface markers that bind to and present foreign antigens to T cells.

MHC molecules are classified as either class I or class II. Class I MHC molecules (MHC I) are expressed on the surface of almost all cells and are involved in the presentation of antigen to cytotoxic T cells. For example, a cell infected with virus will degrade intracellular viral proteins and express the protein fragments bound to MHC I molecules on the cell surface. The MHC I/antigen complex is recognized by cytotoxic T-cells which destroy the infected cell and the virus within. Class II MHC molecules are expressed primarily on specialized antigen-presenting cells of the immune system, such as B-cells and macrophages. These cells ingest foreign proteins from the extracellular fluid and express MHC II/antigen complex on the cell surface. This complex activates helper T-cells, which then secrete cytokines and other factors that stimulate the immune response. MHC molecules also play an important role in organ rejection following transplantation. Rejection occurs when the recipient's T-cells respond to foreign MHC molecules on the transplanted organ in the same way as to self MHC molecules bound to foreign antigen. (Reviewed in Alberts, B. et al. (1994) Molecular Biology of the Cell, Garland Publishing, New York NY, pp. 1229-1246.)

Antibodies, or immunoglobulins, are either expressed on the surface of B-cells or secreted by B-cells into the circulation. Antibodies bind and neutralize foreign antigens in the blood and other extracellular fluids. The prototypical antibody is a tetramer consisting of two identical heavy polypeptide chains (H-chains) and two identical light polypeptide chains (L-chains) interlinked by disulfide bonds. This arrangement confers the characteristic Y-shape to antibody molecules. Antibodies are classified based on their H-chain composition. The five antibody classes, IgA, IgD, IgE, IgG and IgM, are defined by the  $\alpha$ ,  $\delta$ ,  $\epsilon$ ,  $\gamma$ , and  $\mu$  H-chain types. There are two types of L-chains,  $\kappa$  and  $\lambda$ , either of which may associate as a pair with any H-chain pair. IgG, the most common class of antibody found in the circulation, is tetrameric, while the other classes of antibodies are generally variants or multimers of this basic structure.

H-chains and L-chains each contain an N-terminal variable region and a C-terminal constant region. The constant region consists of about 110 amino acids in L-chains and about 330 or 440 amino acids in H-chains. The amino acid sequence of the constant region is nearly identical among H- or L-chains of a particular class. The variable region consists of about 110 amino acids in both H- and L-chains. However, the amino acid sequence of the variable region differs among H- or L-chains of a particular class. Within each H- or L-chain variable region are three hypervariable regions of extensive sequence diversity, each consisting of about 5 to 10 amino acids. In the antibody molecule, the H- and L-chain hypervariable regions come together to form the antigen recognition site. (Reviewed in Alberts, supra, pp. 1206-1213 and 1216-1217.)

Both H-chains and L-chains contain repeated Ig domains. For example, a typical H-chain contains four Ig domains, three of which occur within the constant region and one of which occurs within the variable region and contributes to the formation of the antigen recognition site. Likewise,

a typical L-chain contains two Ig domains, one of which occurs within the constant region and one of which occurs within the variable region.

The immune system is capable of recognizing and responding to any foreign molecule that enters the body. Therefore, the immune system must be armed with a full repertoire of antibodies against all potential antigens. Such antibody diversity is generated by somatic rearrangement of gene segments encoding variable and constant regions. These gene segments are joined together by site-specific recombination which occurs between highly conserved DNA sequences that flank each gene segment. Because there are hundreds of different gene segments, millions of unique genes can be generated combinatorially. In addition, imprecise joining of these segments and an unusually high rate of somatic mutation within these segments further contribute to the generation of a diverse antibody population.

T-cell receptors are both structurally and functionally related to antibodies. (Reviewed in Alberts, supra, pp. 1228-1229.) T-cell receptors are cell surface proteins that bind foreign antigens and mediate diverse aspects of the immune response. A typical T-cell receptor is a heterodimer comprised of two disulfide-linked polypeptide chains called  $\alpha$  and  $\beta$ . Each chain is about 280 amino acids in length and contains one variable region and one constant region. Each variable or constant region folds into an Ig domain. The variable regions from the  $\alpha$  and  $\beta$  chains come together in the heterodimer to form the antigen recognition site. T-cell receptor diversity is generated by somatic rearrangement of gene segments encoding the  $\alpha$  and  $\beta$  chains. T-cell receptors recognize small peptide antigens that are expressed on the surface of antigen-presenting cells and pathogen-infected cells. These peptide antigens are presented on the cell surface in association with major histocompatibility proteins which provide the proper context for antigen recognition.

#### Secreted and Extracellular Matrix Molecules

SEQ ID NO:25 encodes, for example, a secreted/extracellular matrix molecule.

Protein secretion is essential for cellular function. Protein secretion is mediated by a signal peptide located at the amino terminus of the protein to be secreted. The signal peptide is comprised of about ten to twenty hydrophobic amino acids which target the nascent protein from the ribosome to the endoplasmic reticulum (ER). Proteins targeted to the ER may either proceed through the secretory pathway or remain in any of the secretory organelles such as the ER, Golgi apparatus, or lysosomes. Proteins that transit through the secretory pathway are either secreted into the extracellular space or retained in the plasma membrane. Secreted proteins are often synthesized as inactive precursors that are activated by post-translational processing events during transit through the secretory pathway. Such events include glycosylation, proteolysis, and removal of the signal peptide by a signal peptidase. Other events that may occur during protein transport include chaperone-dependent unfolding and



folding of the nascent protein and interaction of the protein with a receptor or pore complex. Examples of secreted proteins with amino terminal signal peptides include receptors, extracellular matrix molecules, cytokines, hormones, growth and differentiation factors, neuropeptides, vasomediators, ion channels, transporters/pumps, and proteases. (Reviewed in Alberts, B. et al. (1994) Molecular Biology of The Cell, Garland Publishing, New York NY, pp. 557-560, 582-592.)

The extracellular matrix (ECM) is a complex network of glycoproteins, polysaccharides, proteoglycans, and other macromolecules that are secreted from the cell into the extracellular space. The ECM remains in close association with the cell surface and provides a supportive meshwork that profoundly influences cell shape, motility, strength, flexibility, and adhesion. In fact, adhesion of a cell to its surrounding matrix is required for cell survival except in the case of metastatic tumor cells, which have overcome the need for cell-ECM anchorage. This phenomenon suggests that the ECM plays a critical role in the molecular mechanisms of growth control and metastasis. (Reviewed in Ruoslahti, E. (1996) *Sci. Am.* 275:72-77.) Furthermore, the ECM determines the structure and physical properties of connective tissue and is particularly important for morphogenesis and other processes associated with embryonic development and pattern formation.

The collagens comprise a family of ECM proteins that provide structure to bone, teeth, skin, ligaments, tendons, cartilage, blood vessels, and basement membranes. Multiple collagen proteins have been identified. Three collagen molecules fold together in a triple helix stabilized by interchain disulfide bonds. Bundles of these triple helices then associate to form fibrils. Collagen primary structure consists of hundreds of (Gly-X-Y) repeats where about a third of the X and Y residues are Pro. Glycines are crucial to helix formation as the bulkier amino acid sidechains cannot fold into the triple helical conformation. Because of these strict sequence requirements, mutations in collagen genes have severe consequences. Osteogenesis imperfecta patients have brittle bones that fracture easily; in severe cases patients die in utero or at birth. Ehlers-Danlos syndrome patients have hyperelastic skin, hypermobile joints, and susceptibility to aortic and intestinal rupture. Chondrodysplasia patients have short stature and ocular disorders. Alport syndrome patients have hematuria, sensorineural deafness, and eye lens deformation. (Isselbacher, K.J. et al. (1994) Harrison's Principles of Internal Medicine, McGraw-Hill, Inc., New York NY, pp. 2105-2117; and Creighton, T.E. (1984) Proteins, Structures and Molecular Principles, W.H. Freeman and Company, New York NY, pp. 191-197.)

Elastin and related proteins confer elasticity to tissues such as skin, blood vessels, and lungs. Elastin is a highly hydrophobic protein of about 750 amino acids that is rich in proline and glycine residues. Elastin molecules are highly cross-linked, forming an extensive extracellular network of fibers and sheets. Elastin fibers are surrounded by a sheath of microfibrils which are composed of a number of glycoproteins, including fibrillin. Mutations in the gene encoding fibrillin are responsible for Marfan's syndrome, a genetic disorder characterized by defects in connective tissue. In severe cases,

the aortas of afflicted individuals are prone to rupture. (Reviewed in Alberts, supra, pp. 984-986.)

Fibronectin is a large ECM glycoprotein found in all vertebrates. Fibronectin exists as a dimer of two subunits, each containing about 2,500 amino acids. Each subunit folds into a rod-like structure containing multiple domains. The domains each contain multiple repeated modules, the most common of which is the type III fibronectin repeat. The type III fibronectin repeat is about 90 amino acids in length and is also found in other ECM proteins and in some plasma membrane and cytoplasmic proteins. Furthermore, some type III fibronectin repeats contain a characteristic tripeptide consisting of Arginine-Glycine-Aspartic acid (RGD). The RGD sequence is recognized by the integrin family of cell surface receptors and is also found in other ECM proteins. Disruption of both copies of the gene encoding fibronectin causes early embryonic lethality in mice. The mutant embryos display extensive morphological defects, including defects in the formation of the notochord, somites, heart, blood vessels, neural tube, and extraembryonic structures. (Reviewed in Alberts, supra, pp. 986-987.)

Laminin is a major glycoprotein component of the basal lamina which underlies and supports epithelial cell sheets. Laminin is one of the first ECM proteins synthesized in the developing embryo. Laminin is an 850 kilodalton protein composed of three polypeptide chains joined in the shape of a cross by disulfide bonds. Laminin is especially important for angiogenesis and in particular, for guiding the formation of capillaries. (Reviewed in Alberts, supra, pp. 990-991.)

There are many other types of proteinaceous ECM components, most of which can be classified as proteoglycans. Proteoglycans are composed of unbranched polysaccharide chains (glycosaminoglycans) attached to protein cores. Common proteoglycans include aggrecan, betaglycan, decorin, perlecan, serglycin, and syndecan-1. Some of these molecules not only provide mechanical support, but also bind to extracellular signaling molecules, such as fibroblast growth factor and transforming growth factor  $\beta$ , suggesting a role for proteoglycans in cell-cell communication and cell growth. (Reviewed in Alberts, supra, pp. 973-978.) Likewise, the glycoproteins tenascin-C and tenascin-R are expressed in developing and lesioned neural tissue and provide stimulatory and anti-adhesive (inhibitory) properties, respectively, for axonal growth. (Faissner, A. (1997) Cell Tissue Res. 290:331-341.)

#### Cytoskeletal Molecules

SEQ ID NO:26 and SEQ ID NO:27 encode, for example, cytoskeletal molecules.

The cytoskeleton is a cytoplasmic network of protein fibers that mediate cell shape, structure, and movement. The cytoskeleton supports the cell membrane and forms tracks along which organelles and other elements move in the cytosol. The cytoskeleton is a dynamic structure that allows cells to adopt various shapes and to carry out directed movements. Major cytoskeletal fibers include the microtubules, the microfilaments, and the intermediate filaments. Motor proteins,

including myosin, dynein, and kinesin, drive movement of or along the fibers. The motor protein dynamin drives the formation of membrane vesicles. Accessory or associated proteins modify the structure or activity of the fibers while cytoskeletal membrane anchors connect the fibers to the cell membrane.

## 5 Tubulins

Microtubules, cytoskeletal fibers with a diameter of about 24 nm, have multiple roles in the cell. Bundles of microtubules form cilia and flagella, which are whip-like extensions of the cell membrane that are necessary for sweeping materials across an epithelium and for swimming of sperm, respectively. Marginal bands of microtubules in red blood cells and platelets are important for  
10 these cells' pliability. Organelles, membrane vesicles, and proteins are transported in the cell along tracks of microtubules. For example, microtubules run through nerve cell axons, allowing bi-directional transport of materials and membrane vesicles between the cell body and the nerve terminal. Failure to supply the nerve terminal with these vesicles blocks the transmission of neural signals. Microtubules are also critical to chromosomal movement during cell division. Both stable  
15 and short-lived populations of microtubules exist in the cell.

Microtubules are polymers of GTP-binding tubulin protein subunits. Each subunit is a heterodimer of  $\alpha$ - and  $\beta$ - tubulin, multiple isoforms of which exist. The hydrolysis of GTP is linked to the addition of tubulin subunits at the end of a microtubule. The subunits interact head to tail to form protofilaments; the protofilaments interact side to side to form a microtubule. A microtubule is  
20 polarized, one end ringed with  $\alpha$ -tubulin and the other with  $\beta$ -tubulin, and the two ends differ in their rates of assembly. Generally, each microtubule is composed of 13 protofilaments although 11 or 15 protofilament-microtubules are sometimes found. Cilia and flagella contain doublet microtubules. Microtubules grow from specialized structures known as centrosomes or microtubule-organizing centers (MTOCs). MTOCs may contain one or two centrioles, which are pinwheel arrays of triplet  
25 microtubules. The basal body, the organizing center located at the base of a cilium or flagellum, contains one centriole. Gamma tubulin present in the MTOC is important for nucleating the polymerization of  $\alpha$ - and  $\beta$ - tubulin heterodimers but does not polymerize into microtubules.

## Microtubule-Associated Proteins

Microtubule-associated proteins (MAPs) have roles in the assembly and stabilization of  
30 microtubules. One major family of MAPs, assembly MAPs, can be identified in neurons as well as non-neuronal cells. Assembly MAPs are responsible for cross-linking microtubules in the cytosol. These MAPs are organized into two domains: a basic microtubule-binding domain and an acidic projection domain. The projection domain is the binding site for membranes, intermediate filaments, or other microtubules. Based on sequence analysis, assembly MAPs can be further grouped into two  
35 types: Type I and Type II. Type I MAPs, which include MAP1A and MAP1B, are large, filamentous

molecules that co-purify with microtubules and are abundantly expressed in brain and testes. Type I MAPs contain several repeats of a positively-charged amino acid sequence motif that binds and neutralizes negatively charged tubulin, leading to stabilization of microtubules. MAP1A and MAP1B are each derived from a single precursor polypeptide that is subsequently proteolytically processed to generate one heavy chain and one light chain.

Another light chain, LC3, is a 16.4 kDa molecule that binds MAP1A, MAP1B, and microtubules. It is suggested that LC3 is synthesized from a source other than the MAP1A or MAP1B transcripts, and that the expression of LC3 may be important in regulating the microtubule binding activity of MAP1A and MAP1B during cell proliferation (Mann, S.S. et al. (1994) J. Biol. Chem. 269:11492-11497).

Type II MAPs, which include MAP2a, MAP2b, MAP2c, MAP4, and Tau, are characterized by three to four copies of an 18-residue sequence in the microtubule-binding domain. MAP2a, MAP2b, and MAP2c are found only in dendrites, MAP4 is found in non-neuronal cells, and Tau is found in axons and dendrites of nerve cells. Alternative splicing of the Tau mRNA leads to the existence of multiple forms of Tau protein. Tau phosphorylation is altered in neurodegenerative disorders such as Alzheimer's disease, Pick's disease, progressive supranuclear palsy, corticobasal degeneration, and familial frontotemporal dementia and Parkinsonism linked to chromosome 17. The altered Tau phosphorylation leads to a collapse of the microtubule network and the formation of intraneuronal Tau aggregates (Spillantini, M.G. and M. Goedert (1998) Trends Neurosci. 21:428-433).

The protein pericentrin is found in the MTOC and has a role in microtubule assembly.

### Actins

Microfilaments, cytoskeletal filaments with a diameter of about 7-9 nm, are vital to cell locomotion, cell shape, cell adhesion, cell division, and muscle contraction. Assembly and disassembly of the microfilaments allow cells to change their morphology. Microfilaments are the polymerized form of actin, the most abundant intracellular protein in the eukaryotic cell. Human cells contain six isoforms of actin. The three  $\alpha$ -actins are found in different kinds of muscle, nonmuscle  $\beta$ -actin and nonmuscle  $\gamma$ -actin are found in nonmuscle cells, and another  $\gamma$ -actin is found in intestinal smooth muscle cells. G-actin, the monomeric form of actin, polymerizes into polarized, helical F-actin filaments, accompanied by the hydrolysis of ATP to ADP. Actin filaments associate to form bundles and networks, providing a framework to support the plasma membrane and determine cell shape. These bundles and networks are connected to the cell membrane. In muscle cells, thin filaments containing actin slide past thick filaments containing the motor protein myosin during contraction. A family of actin-related proteins exist that are not part of the actin cytoskeleton, but rather associate with microtubules and dynein.

### Actin-Associated Proteins

Actin-associated proteins have roles in cross-linking, severing, and stabilization of actin filaments and in sequestering actin monomers. Several of the actin-associated proteins have multiple functions. Bundles and networks of actin filaments are held together by actin cross-linking proteins. These proteins have two actin-binding sites, one for each filament. Short cross-linking proteins promote bundle formation while longer, more flexible cross-linking proteins promote network formation. Calmodulin-like calcium-binding domains in actin cross-linking proteins allow calcium regulation of cross-linking. Group I cross-linking proteins have unique actin-binding domains and include the 30 kD protein, EF-1a, fascin, and scruin. Group II cross-linking proteins have a 7,000-MW actin-binding domain and include villin and dematin. Group III cross-linking proteins have pairs of a 26,000-MW actin-binding domain and include fimbrin, spectrin, dystrophin, ABP 120, and filamin.

Severing proteins regulate the length of actin filaments by breaking them into short pieces or by blocking their ends. Severing proteins include gCAP39, severin (fragmin), gelsolin, and villin. Capping proteins can cap the ends of actin filaments, but cannot break filaments. Capping proteins include CapZ and tropomodulin. The proteins thymosin and profilin sequester actin monomers in the cytosol, allowing a pool of unpolymerized actin to exist. The actin-associated proteins tropomyosin, troponin, and caldesmon regulate muscle contraction in response to calcium.

#### Intermediate Filaments and Associated Proteins

Intermediate filaments (IFs) are cytoskeletal fibers with a diameter of about 10 nm, intermediate between that of microfilaments and microtubules. IFs serve structural roles in the cell, reinforcing cells and organizing cells into tissues. IFs are particularly abundant in epidermal cells and in neurons. IFs are extremely stable, and, in contrast to microfilaments and microtubules, do not function in cell motility.

Five types of IF proteins are known in mammals. Type I and Type II proteins are the acidic and basic keratins, respectively. Heterodimers of the acidic and basic keratins are the building blocks of keratin IFs. Keratins are abundant in soft epithelia such as skin and cornea, hard epithelia such as nails and hair, and in epithelia that line internal body cavities. Mutations in keratin genes lead to epithelial diseases including epidermolysis bullosa simplex, bullous congenital ichthyosiform erythroderma (epidermolytic hyperkeratosis), non-epidermolytic and epidermolytic palmoplantar keratoderma, ichthyosis bullosa of Siemens, pachyonychia congenita, and white sponge nevus. Some of these diseases result in severe skin blistering. (See, e.g., Wawersik, M. et al. (1997) J. Biol. Chem. 272:32557-32565; and Corden L.D. and W.H. McLean (1996) Exp. Dermatol. 5:297-307.)

Type III IF proteins include desmin, glial fibrillary acidic protein, vimentin, and peripherin. Desmin filaments in muscle cells link myofibrils into bundles and stabilize sarcomeres in contracting muscle. Glial fibrillary acidic protein filaments are found in the glial cells that surround neurons and

astrocytes. Vimentin filaments are found in blood vessel endothelial cells, some epithelial cells, and mesenchymal cells such as fibroblasts, and are commonly associated with microtubules. Vimentin filaments may have roles in keeping the nucleus and other organelles in place in the cell. Type IV IFs include the neurofilaments and nestin. Neurofilaments, composed of three polypeptides NF-L, NF-M, and NF-H, are frequently associated with microtubules in axons. Neurofilaments are responsible for the radial growth and diameter of an axon, and ultimately for the speed of nerve impulse transmission. Changes in phosphorylation and metabolism of neurofilaments are observed in neurodegenerative diseases including amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease (Julien, J.P. and W.E. Mushynski (1998) *Prog. Nucleic Acid Res. Mol. Biol.* 61:1-23). Type V IFs, the lamins, are found in the nucleus where they support the nuclear membrane.

IFs have a central  $\alpha$ -helical rod region interrupted by short nonhelical linker segments. The rod region is bracketed, in most cases, by non-helical head and tail domains. The rod regions of intermediate filament proteins associate to form a coiled-coil dimer. A highly ordered assembly process leads from the dimers to the IFs. Neither ATP nor GTP is needed for IF assembly, unlike that of microfilaments and microtubules.

IF-associated proteins (IFAPs) mediate the interactions of IFs with one another and with other cell structures. IFAPs cross-link IFs into a bundle, into a network, or to the plasma membrane, and may cross-link IFs to the microfilament and microtubule cytoskeleton. Microtubules and IFs are in particular closely associated. IFAPs include BPAG1, plakoglobin, desmoplakin I, desmoplakin II, plectin, ankyrin, filaggrin, and lamin B receptor.

#### Cytoskeletal-Membrane Anchors

Cytoskeletal fibers are attached to the plasma membrane by specific proteins. These attachments are important for maintaining cell shape and for muscle contraction. In erythrocytes, the spectrin-actin cytoskeleton is attached to cell membrane by three proteins, band 4.1, ankyrin, and adducin. Defects in this attachment result in abnormally shaped cells which are more rapidly degraded by the spleen, leading to anemia. In platelets, the spectrin-actin cytoskeleton is also linked to the membrane by ankyrin; a second actin network is anchored to the membrane by filamin. In muscle cells the protein dystrophin links actin filaments to the plasma membrane; mutations in the dystrophin gene lead to Duchenne muscular dystrophy. In adherens junctions and adhesion plaques the peripheral membrane proteins  $\alpha$ -actinin and vinculin attach actin filaments to the cell membrane.

IFs are also attached to membranes by cytoskeletal-membrane anchors. The nuclear lamina is attached to the inner surface of the nuclear membrane by the lamin B receptor. Vimentin IFs are attached to the plasma membrane by ankyrin and plectin. Desmosome and hemidesmosome membrane junctions hold together epithelial cells of organs and skin. These membrane junctions allow shear forces to be distributed across the entire epithelial cell layer, thus providing strength and

rigidity to the epithelium. IFs in epithelial cells are attached to the desmosome by plakoglobin and desmoplakins. The proteins that link IFs to hemidesmosomes are not known. Desmin IFs surround the sarcomere in muscle and are linked to the plasma membrane by paranemin, synemin, and ankyrin.

#### Myosin-related Motor Proteins

5 Myosins are actin-activated ATPases, found in eukaryotic cells, that couple hydrolysis of ATP with motion. Myosin provides the motor function for muscle contraction and intracellular movements such as phagocytosis and rearrangement of cell contents during mitotic cell division (cytokinesis). The contractile unit of skeletal muscle, termed the sarcomere, consists of highly ordered arrays of thin actin-containing filaments and thick myosin-containing filaments. Crossbridges form  
10 between the thick and thin filaments, and the ATP-dependent movement of myosin heads within the thick filaments pulls the thin filaments, shortening the sarcomere and thus the muscle fiber.

Myosins are composed of one or two heavy chains and associated light chains. Myosin heavy chains contain an amino-terminal motor or head domain, a neck that is the site of light-chain binding, and a carboxy-terminal tail domain. The tail domains may associate to form an  $\alpha$ -helical  
15 coiled coil. Conventional myosins, such as those found in muscle tissue, are composed of two myosin heavy-chain subunits, each associated with two light-chain subunits that bind at the neck region and play a regulatory role. Unconventional myosins, believed to function in intracellular motion, may contain either one or two heavy chains and associated light chains. There is evidence for about 25 myosin heavy chain genes in vertebrates, more than half of them unconventional.

#### 20 Dynein-related Motor Proteins

Dyneins are (-) end-directed motor proteins which act on microtubules. Two classes of dyneins, cytosolic and axonemal, have been identified. Cytosolic dyneins are responsible for translocation of materials along cytoplasmic microtubules, for example, transport from the nerve terminal to the cell body and transport of endocytic vesicles to lysosomes. Cytoplasmic dyneins are  
25 also reported to play a role in mitosis. Axonemal dyneins are responsible for the beating of flagella and cilia. Dynein on one microtubule doublet walks along the adjacent microtubule doublet. This sliding force produces bending forces that cause the flagellum or cilium to beat. Dyneins have a native mass between 1000 and 2000 kDa and contain either two or three force-producing heads driven by the hydrolysis of ATP. The heads are linked via stalks to a basal domain which is composed of a highly  
30 variable number of accessory intermediate and light chains.

#### Kinesin-related Motor Proteins

Kinesins are (+) end-directed motor proteins which act on microtubules. The prototypical kinesin molecule is involved in the transport of membrane-bound vesicles and organelles. This function is particularly important for axonal transport in neurons. Kinesin is also important in all cell types for  
35 the transport of vesicles from the Golgi complex to the endoplasmic reticulum. This role is critical for

maintaining the identity and functionality of these secretory organelles.

Kinesins define a ubiquitous, conserved family of over 50 proteins that can be classified into at least 8 subfamilies based on primary amino acid sequence, domain structure, velocity of movement, and cellular function. (Reviewed in Moore, J.D. and S.A. Endow (1996) Bioessays 18:207-219; and Hoyt, A.M. (1994) Curr. Opin. Cell Biol. 6:63-68.) The prototypical kinesin molecule is a heterotetramer comprised of two heavy polypeptide chains (KHCs) and two light polypeptide chains (KLCs). The KHC subunits are typically referred to as "kinesin." KHC is about 1000 amino acids in length, and KLC is about 550 amino acids in length. Two KHCs dimerize to form a rod-shaped molecule with three distinct regions of secondary structure. At one end of the molecule is a globular motor domain that functions in ATP hydrolysis and microtubule binding. Kinesin motor domains are highly conserved and share over 70% identity. Beyond the motor domain is an  $\alpha$ -helical coiled-coil region which mediates dimerization. At the other end of the molecule is a fan-shaped tail that associates with molecular cargo. The tail is formed by the interaction of the KHC C-termini with the two KLCs.

Members of the more divergent subfamilies of kinesins are called kinesin-related proteins (KRPs), many of which function during mitosis in eukaryotes (Hoyt, supra). Some KRPs are required for assembly of the mitotic spindle. In vivo and in vitro analyses suggest that these KRPs exert force on microtubules that comprise the mitotic spindle, resulting in the separation of spindle poles. Phosphorylation of KRP is required for this activity. Failure to assemble the mitotic spindle results in abortive mitosis and chromosomal aneuploidy, the latter condition being characteristic of cancer cells. In addition, a unique KRP, centromere protein E, localizes to the kinetochore of human mitotic chromosomes and may play a role in their segregation to opposite spindle poles.

#### Dynamin-related Motor Proteins

Dynamin is a large GTPase motor protein that functions as a "molecular pinchase," generating a mechanochemical force used to sever membranes. This activity is important in forming clathrin-coated vesicles from coated pits in endocytosis and in the biogenesis of synaptic vesicles in neurons. Binding of dynamin to a membrane leads to dynamin's self-assembly into spirals that may act to constrict a flat membrane surface into a tubule. GTP hydrolysis induces a change in conformation of the dynamin polymer that pinches the membrane tubule, leading to severing of the membrane tubule and formation of a membrane vesicle. Release of GDP and inorganic phosphate leads to dynamin disassembly. Following disassembly the dynamin may either dissociate from the membrane or remain associated to the vesicle and be transported to another region of the cell. Three homologous dynamin genes have been discovered, in addition to several dynamin-related proteins. Conserved dynamin regions are the N-terminal GTP-binding domain, a central pleckstrin homology domain that binds membranes, a central coiled-coil region that may activate dynamin's GTPase activity, and a C-terminal proline-rich domain that contains several motifs that bind SH3 domains on



other proteins. Some dynamin-related proteins do not contain the pleckstrin homology domain or the proline-rich domain. (See McNiven, M.A. (1998) Cell 94:151-154; Scaife, R.M. and R.L. Margolis (1997) Cell. Signal. 9:395-401.)

The cytoskeleton is reviewed in Lodish, H. et al. (1995) Molecular Cell Biology, Scientific American Books, New York NY.

### Ribosomal Molecules

SEQ ID NO:30 and SEQ ID NO:31 encode, for example, ribosomal molecules.

Ribosomal RNAs (rRNAs) are assembled, along with ribosomal proteins, into ribosomes, which are cytoplasmic particles that translate messenger RNA into polypeptides. The eukaryotic ribosome is composed of a 60S (large) subunit and a 40S (small) subunit, which together form the 80S ribosome. In addition to the 18S, 28S, 5S, and 5.8S rRNAs, the ribosome also contains more than fifty proteins. The ribosomal proteins have a prefix which denotes the subunit to which they belong, either L (large) or S (small). Ribosomal protein activities include binding rRNA and organizing the conformation of the junctions between rRNA helices (Woodson, S.A. and N.B. Leontis (1998) Curr. Opin. Struct. Biol. 8:294-300; Ramakrishnan, V. and S.W. White (1998) Trends Biochem. Sci. 23:208-212.) Three important sites are identified on the ribosome. The aminoacyl-tRNA site (A site) is where charged tRNAs (with the exception of the initiator-tRNA) bind on arrival at the ribosome. The peptidyl-tRNA site (P site) is where new peptide bonds are formed, as well as where the initiator tRNA binds. The exit site (E site) is where deacylated tRNAs bind prior to their release from the ribosome. (The ribosome is reviewed in Stryer, L. (1995) Biochemistry W.H. Freeman and Company, New York NY, pp. 888-908; and Lodish, H. et al. (1995) Molecular Cell Biology Scientific American Books, New York NY, pp. 119-138.)

### Chromatin Molecules

The nuclear DNA of eukaryotes is organized into chromatin. Two types of chromatin are observed: euchromatin, some of which may be transcribed, and heterochromatin so densely packed that much of it is inaccessible to transcription. Chromatin packing thus serves to regulate protein expression in eukaryotes. Bacteria lack chromatin and the chromatin-packing level of gene regulation.

The fundamental unit of chromatin is the nucleosome of 200 DNA base pairs associated with two copies each of histones H2A, H2B, H3, and H4. Adjacent nucleosomes are linked by another class of histones, H1. Low molecular weight non-histone proteins called the high mobility group (HMG), associated with chromatin, may function in the unwinding of DNA and stabilization of single-stranded DNA. Chromodomain proteins function in compaction of chromatin into its transcriptionally silent heterochromatin form.

During mitosis, all DNA is compacted into heterochromatin and transcription ceases. Transcription in interphase begins with the activation of a region of chromatin. Active chromatin is decondensed. Decondensation appears to be accompanied by changes in binding coefficient, phosphorylation and acetylation states of chromatin histones. HMG proteins HMG13 and HMG17 selectively bind activated chromatin. Topoisomerases remove superhelical tension on DNA. The activated region decondenses, allowing gene regulatory proteins and transcription factors to assemble on the DNA.

Patterns of chromatin structure can be stably inherited, producing heritable patterns of gene expression. In mammals, one of the two X chromosomes in each female cell is inactivated by condensation to heterochromatin during zygote development. The inactive state of this chromosome is inherited, so that adult females are mosaics of clusters of paternal-X and maternal-X clonal cell groups. The condensed X chromosome is reactivated in meiosis.

Chromatin is associated with disorders of protein expression such as thalassemia, a genetic anemia resulting from the removal of the locus control region (LCR) required for decondensation of the globin gene locus.

For a review of chromatin structure and function see Alberts, B. et al. (1994) Molecular Cell Biology, third edition, Garland Publishing, Inc., New York NY, pp. 351-354, 433-439.

#### Electron Transfer Associated Molecules

SEQ ID NO:23 and SEQ ID NO:24 encode, for example, electron transfer associated molecules.

Electron carriers such as cytochromes accept electrons from NADH or FADH<sub>2</sub> and donate them to other electron carriers. Most electron-transferring proteins, except ubiquinone, are prosthetic groups such as flavins, heme, FeS clusters, and copper, bound to inner membrane proteins.

Adrenodoxin, for example, is an FeS protein that forms a complex with NADPH:adrenodoxin reductase and cytochrome p450. Cytochromes contain a heme prosthetic group, a porphyrin ring containing a tightly bound iron atom. Electron transfer reactions play a crucial role in cellular energy production.

Energy is produced by the oxidation of glucose and fatty acids. Glucose is initially converted to pyruvate in the cytoplasm. Fatty acids and pyruvate are transported to the mitochondria for complete oxidation to CO<sub>2</sub> coupled by enzymes to the transport of electrons from NADH and FADH<sub>2</sub> to oxygen and to the synthesis of ATP (oxidative phosphorylation) from ADP and P<sub>i</sub>.

Pyruvate is transported into the mitochondria and converted to acetyl-CoA for oxidation via the citric acid cycle, involving pyruvate dehydrogenase components, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase. Enzymes involved in the citric acid cycle include: citrate synthetase,

aconitases, isocitrate dehydrogenase, alpha-ketoglutarate dehydrogenase complex including transsuccinylases, succinyl CoA synthetase, succinate dehydrogenase, fumarases, and malate dehydrogenase. Acetyl CoA is oxidized to CO<sub>2</sub> with concomitant formation of NADH, FADH<sub>2</sub>, and GTP. In oxidative phosphorylation, the transfer of electrons from NADH and FADH<sub>2</sub> to oxygen by dehydrogenases is coupled to the synthesis of ATP from ADP and P<sub>i</sub> by the F<sub>0</sub>F<sub>1</sub> ATPase complex in the mitochondrial inner membrane. Enzyme complexes responsible for electron transport and ATP synthesis include the F<sub>0</sub>F<sub>1</sub> ATPase complex, ubiquinone(CoQ)-cytochrome c reductase, ubiquinone reductase, cytochrome b, cytochrome c<sub>1</sub>, FeS protein, and cytochrome c oxidase.

ATP synthesis requires membrane transport enzymes including the phosphate transporter and the ATP-ADP antiport protein. The ATP-binding cassette (ABC) superfamily has also been suggested as belonging to the mitochondrial transport group (Hogue, D.L. et al. (1999) J. Mol. Biol. 285:379-389). Brown fat uncoupling protein dissipates oxidative energy as heat, and may be involved the fever response to infection and trauma (Cannon, B. et al. (1998) Ann. NY Acad. Sci. 856:171-187).

Mitochondria are oval-shaped organelles comprising an outer membrane, a tightly folded inner membrane, an intermembrane space between the outer and inner membranes, and a matrix inside the inner membrane. The outer membrane contains many porin molecules that allow ions and charged molecules to enter the intermembrane space, while the inner membrane contains a variety of transport proteins that transfer only selected molecules. Mitochondria are the primary sites of energy production in cells.

Mitochondria contain a small amount of DNA. Human mitochondrial DNA encodes 13 proteins, 22 tRNAs, and 2 rRNAs. Mitochondrial-DNA encoded proteins include NADH-Q reductase, a cytochrome reductase subunit, cytochrome oxidase subunits, and ATP synthase subunits.

Electron-transfer reactions also occur outside the mitochondria in locations such as the endoplasmic reticulum, which plays a crucial role in lipid and protein biosynthesis. Cytochrome b5 is a central electron donor for various reductive reactions occurring on the cytoplasmic surface of liver endoplasmic reticulum. Cytochrome b5 has been found in Golgi, plasma, endoplasmic reticulum (ER), and microbody membranes.

For a review of mitochondrial metabolism and regulation, see Lodish, H. et al. (1995) Molecular Cell Biology, Scientific American Books, New York NY, pp. 745-797 and Stryer (1995) Biochemistry, W.H. Freeman and Co., San Francisco CA., pp 529-558, 988-989.

The majority of mitochondrial proteins are encoded by nuclear genes, are synthesized on cytosolic ribosomes, and are imported into the mitochondria. Nuclear-encoded proteins which are destined for the mitochondrial matrix typically contain positively-charged amino terminal signal sequences. Import of these preproteins from the cytoplasm requires a multisubunit protein complex in the outer membrane known as the translocase of outer mitochondrial membrane (TOM; previously

designated MOM; Pfanner, N. et al. (1996) Trends Biochem. Sci. 21:51-52) and at least three inner membrane proteins which comprise the translocase of inner mitochondrial membrane (TIM; previously designated MIM; Pfanner, supra). An inside-negative membrane potential across the inner mitochondrial membrane is also required for preprotein import. Preproteins are recognized by surface receptor components of the TOM complex and are translocated through a proteinaceous pore formed by other TOM components. Proteins targeted to the matrix are then recognized by the import machinery of the TIM complex. The import systems of the outer and inner membranes can function independently (Segui-Real, B. et al. (1993) EMBO J. 12:2211-2218).

Once precursor proteins are in the mitochondria, the leader peptide is cleaved by a signal peptidase to generate the mature protein. Most leader peptides are removed in a one step process by a protease termed mitochondrial processing peptidase (MPP) (Paces, V. et al. (1993) Proc. Natl. Acad. Sci. USA 90:5355-5358). In some cases a two-step process occurs in which MPP generates an intermediate precursor form which is cleaved by a second enzyme, mitochondrial intermediate peptidase, to generate the mature protein.

Mitochondrial dysfunction leads to impaired calcium buffering, generation of free radicals that may participate in deleterious intracellular and extracellular processes, changes in mitochondrial permeability and oxidative damage which is observed in several neurodegenerative diseases. Neurodegenerative diseases linked to mitochondrial dysfunction include some forms of Alzheimer's disease, Friedreich's ataxia, familial amyotrophic lateral sclerosis, and Huntington's disease (Beal, M.F. (1998) Biochim. Biophys. Acta 1366:211-213). The myocardium is heavily dependent on oxidative metabolism, so mitochondrial dysfunction often leads to heart disease (DiMauro, S. and M. Hirano (1998) Curr. Opin. Cardiol 13:190-197). Mitochondria are implicated in disorders of cell proliferation, since they play an important role in a cell's decision to proliferate or self-destruct through apoptosis. The oncoprotein Bcl-2, for example, promotes cell proliferation by stabilizing mitochondrial membranes so that apoptosis signals are not released (Susin, S.A. (1998) Biochim. Biophys. Acta 1366:151-165).

#### Transcription Factor Molecules

SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, and SEQ ID NO:43 encode, for example, transcription factor molecules.

Multicellular organisms are comprised of diverse cell types that differ dramatically both in structure and function. The identity of a cell is determined by its characteristic pattern of gene expression, and different cell types express overlapping but distinctive sets of genes throughout development. Spatial and temporal regulation of gene expression is critical for the control of cell

proliferation, cell differentiation, apoptosis, and other processes that contribute to organismal development. Furthermore, gene expression is regulated in response to extracellular signals that mediate cell-cell communication and coordinate the activities of different cell types. Appropriate gene regulation also ensures that cells function efficiently by expressing only those genes whose

5 functions are required at a given time.

Transcriptional regulatory proteins are essential for the control of gene expression. Some of these proteins function as transcription factors that initiate, activate, repress, or terminate gene transcription. Transcription factors generally bind to the promoter, enhancer, and upstream regulatory regions of a gene in a sequence-specific manner, although some factors bind regulatory elements

10 within or downstream of a gene's coding region. Transcription factors may bind to a specific region of DNA singly or as a complex with other accessory factors. (Reviewed in Lewin, B. (1990) Genes IV, Oxford University Press, New York NY, and Cell Press, Cambridge MA, pp. 554-570.)

The double helix structure and repeated sequences of DNA create topological and chemical features which can be recognized by transcription factors. These features are hydrogen bond donor

15 and acceptor groups, hydrophobic patches, major and minor grooves, and regular, repeated stretches of sequence which induce distinct bends in the helix. Typically, transcription factors recognize specific DNA sequence motifs of about 20 nucleotides in length. Multiple, adjacent transcription factor-binding motifs may be required for gene regulation.

Many transcription factors incorporate DNA-binding structural motifs which comprise either

20  $\alpha$  helices or  $\beta$  sheets that bind to the major groove of DNA. Four well-characterized structural motifs are helix-turn-helix, zinc finger, leucine zipper, and helix-loop-helix. Proteins containing these motifs may act alone as monomers, or they may form homo- or heterodimers that interact with DNA.

The helix-turn-helix motif consists of two  $\alpha$  helices connected at a fixed angle by a short chain of amino acids. One of the helices binds to the major groove. Helix-turn-helix motifs are

25 exemplified by the homeobox motif which is present in homeodomain proteins. These proteins are critical for specifying the anterior-posterior body axis during development and are conserved throughout the animal kingdom. The Antennapedia and Ultrabithorax proteins of Drosophila melanogaster are prototypical homeodomain proteins (Pabo, C.O. and R.T. Sauer (1992) *Annu. Rev. Biochem.* 61:1053-1095).

The zinc finger motif, which binds zinc ions, generally contains tandem repeats of about 30 amino acids consisting of periodically spaced cysteine and histidine residues. Examples of this sequence pattern, designated C2H2 and C3HC4 ("RING" finger), have been described (Lewin, supra). Zinc finger proteins each contain an  $\alpha$  helix and an antiparallel  $\beta$  sheet whose proximity and conformation are maintained by the zinc ion. Contact with DNA is made by the arginine preceding

35 the  $\alpha$  helix and by the second, third, and sixth residues of the  $\alpha$  helix. Variants of the zinc finger

motif include poorly defined cysteine-rich motifs which bind zinc or other metal ions. These motifs may not contain histidine residues and are generally nonrepetitive.

The leucine zipper motif comprises a stretch of amino acids rich in leucine which can form an amphipathic  $\alpha$  helix. This structure provides the basis for dimerization of two leucine zipper proteins. The region adjacent to the leucine zipper is usually basic, and upon protein dimerization, is optimally positioned for binding to the major groove. Proteins containing such motifs are generally referred to as bZIP transcription factors.

The helix-loop-helix motif (HLH) consists of a short  $\alpha$  helix connected by a loop to a longer  $\alpha$  helix. The loop is flexible and allows the two helices to fold back against each other and to bind to DNA. The transcription factor Myc contains a prototypical HLH motif.

Most transcription factors contain characteristic DNA binding motifs, and variations on the above motifs and new motifs have been and are currently being characterized (Faisst, S. and S. Meyer (1992) *Nucleic Acids Res.* 20:3-26).

Many neoplastic disorders in humans can be attributed to inappropriate gene expression.

Malignant cell growth may result from either excessive expression of tumor promoting genes or insufficient expression of tumor suppressor genes (Cleary, M.L. (1992) *Cancer Surv.* 15:89-104). Chromosomal translocations may also produce chimeric loci which fuse the coding sequence of one gene with the regulatory regions of a second unrelated gene. Such an arrangement likely results in inappropriate gene transcription, potentially contributing to malignancy.

In addition, the immune system responds to infection or trauma by activating a cascade of events that coordinate the progressive selection, amplification, and mobilization of cellular defense mechanisms. A complex and balanced program of gene activation and repression is involved in this process. However, hyperactivity of the immune system as a result of improper or insufficient regulation of gene expression may result in considerable tissue or organ damage. This damage is well documented in immunological responses associated with arthritis, allergens, heart attack, stroke, and infections (Isselbacher, K.J. et al. (1996) Harrison's Principles of Internal Medicine, 13/e, McGraw Hill, Inc. and Teton Data Systems Software).

Furthermore, the generation of multicellular organisms is based upon the induction and coordination of cell differentiation at the appropriate stages of development. Central to this process is differential gene expression, which confers the distinct identities of cells and tissues throughout the body. Failure to regulate gene expression during development can result in developmental disorders. Human developmental disorders caused by mutations in zinc finger-type transcriptional regulators include: urogenital developmental abnormalities associated with WT1; Greig cephalopolysyndactyly, Pallister-Hall syndrome, and postaxial polydactyly type A (GLI3); and Townes-Brocks syndrome, characterized by anal, renal, limb, and ear abnormalities (SALL1)

(Engelkamp, D. and V. van Heyningen (1996) *Curr. Opin. Genet. Dev.* 6:334-342; Kohlhasse, J. et al. (1999) *Am. J. Hum. Genet.* 64:435-445).

### Cell Membrane Molecules

5 SEQ ID NO:28 and SEQ ID NO:29 encode, for example, cell membrane molecules.

Eukaryotic cells are surrounded by plasma membranes which enclose the cell and maintain an environment inside the cell that is distinct from its surroundings. In addition, eukaryotic organisms are distinct from prokaryotes in possessing many intracellular organelle and vesicle structures. Many of the metabolic reactions which distinguish eukaryotic biochemistry from prokaryotic biochemistry  
10 take place within these structures. The plasma membrane and the membranes surrounding organelles and vesicles are composed of phosphoglycerides, fatty acids, cholesterol, phospholipids, glycolipids, proteoglycans, and proteins. These components confer identity and functionality to the membranes with which they associate.

### Integral Membrane Proteins

15 The majority of known integral membrane proteins are transmembrane proteins (TM) which are characterized by an extracellular, a transmembrane, and an intracellular domain. TM domains are typically comprised of 15 to 25 hydrophobic amino acids which are predicted to adopt an  $\alpha$ -helical conformation. TM proteins are classified as bitopic (Types I and II) and polytopic (Types III and IV) (Singer, S.J. (1990) *Annu. Rev. Cell Biol.* 6:247-296). Bitopic proteins span the membrane once  
20 while polytopic proteins contain multiple membrane-spanning segments. TM proteins function as cell-surface receptors, receptor-interacting proteins, transporters of ions or metabolites, ion channels, cell anchoring proteins, and cell type-specific surface antigens.

Many membrane proteins (MPs) contain amino acid sequence motifs that target these proteins to specific subcellular sites. Examples of these motifs include PDZ domains, KDEI, RGD, NGR,  
25 and GSL sequence motifs, von Willebrand factor A (vWFA) domains, and EGF-like domains. RGD, NGR, and GSL motif-containing peptides have been used as drug delivery agents in targeted cancer treatment of tumor vasculature (Arap, W. et al. (1998) *Science* 279:377-380). Furthermore, MPs may also contain amino acid sequence motifs, such as the carbohydrate recognition domain (CRD), that mediate interactions with extracellular or intracellular molecules.

### 30 G-Protein Coupled Receptors

G-protein coupled receptors (GPCR) are a superfamily of integral membrane proteins which transduce extracellular signals. GPCRs include receptors for biogenic amines, lipid mediators of inflammation, peptide hormones, and sensory signal mediators. The structure of these highly-conserved receptors consists of seven hydrophobic transmembrane regions, an extracellular  
35 N-terminus, and a cytoplasmic C-terminus. Three extracellular loops alternate with three intracellular

loops to link the seven transmembrane regions. Cysteine disulfide bridges connect the second and third extracellular loops. The most conserved regions of GPCRs are the transmembrane regions and the first two cytoplasmic loops. A conserved, acidic-Arg-aromatic residue triplet present in the second cytoplasmic loop may interact with G proteins. A GPCR consensus pattern is characteristic of most proteins belonging to this superfamily (ExpASY PROSITE document PS00237; and Watson, S. and S. Arkininstall (1994) The G-protein Linked Receptor Facts Book, Academic Press, San Diego CA, pp. 2-6). Mutations and changes in transcriptional activation of GPCR-encoding genes have been associated with neurological disorders such as schizophrenia, Parkinson's disease, Alzheimer's disease, drug addiction, and feeding disorders.

#### 10 Scavenger Receptors

Macrophage scavenger receptors with broad ligand specificity may participate in the binding of low density lipoproteins (LDL) and foreign antigens. Scavenger receptors types I and II are trimeric membrane proteins with each subunit containing a small N-terminal intracellular domain, a transmembrane domain, a large extracellular domain, and a C-terminal cysteine-rich domain. The extracellular domain contains a short spacer region, an  $\alpha$ -helical coiled-coil region, and a triple helical collagen-like region. These receptors have been shown to bind a spectrum of ligands, including chemically modified lipoproteins and albumin, polyribonucleotides, polysaccharides, phospholipids, and asbestos (Matsumoto, A. et al. (1990) Proc. Natl. Acad. Sci. USA 87:9133-9137; and Elomaa, O. et al. (1995) Cell 80:603-609). The scavenger receptors are thought to play a key role in atherogenesis by mediating uptake of modified LDL in arterial walls, and in host defense by binding bacterial endotoxins, bacteria, and protozoa.

#### 20 Tetraspan Family Proteins

The transmembrane 4 superfamily (TM4SF) or tetraspan family is a multigene family encoding type III integral membrane proteins (Wright, M.D. and M.G. Tomlinson (1994) Immunol. Today 15:588-594). The TM4SF is comprised of membrane proteins which traverse the cell membrane four times. Members of the TM4SF include platelet and endothelial cell membrane proteins, melanoma-associated antigens, leukocyte surface glycoproteins, colonic carcinoma antigens, tumor-associated antigens, and surface proteins of the schistosome parasites (Jankowski, S.A. (1994) Oncogene 9:1205-1211). Members of the TM4SF share about 25-30% amino acid sequence identity with one another.

A number of TM4SF members have been implicated in signal transduction, control of cell adhesion, regulation of cell growth and proliferation, including development and oncogenesis, and cell motility, including tumor cell metastasis. Expression of TM4SF proteins is associated with a variety of tumors and the level of expression may be altered when cells are growing or activated.

#### 35 Tumor Antigens



Tumor antigens are cell surface molecules that are differentially expressed in tumor cells relative to normal cells. Tumor antigens distinguish tumor cells immunologically from normal cells and provide diagnostic and therapeutic targets for human cancers (Takagi, S. et al. (1995) *Int. J. Cancer* 61:706-715; Liu, E. et al. (1992) *Oncogene* 7:1027-1032).

#### 5 Leukocyte Antigens

Other types of cell surface antigens include those identified on leukocytic cells of the immune system. These antigens have been identified using systematic, monoclonal antibody (mAb)-based "shotgun" techniques. These techniques have resulted in the production of hundreds of mAbs directed against unknown cell surface leukocytic antigens. These antigens have been grouped into "clusters of differentiation" based on common immunocytochemical localization patterns in various differentiated and undifferentiated leukocytic cell types. Antigens in a given cluster are presumed to identify a single cell surface protein and are assigned a "cluster of differentiation" or "CD" designation. Some of the genes encoding proteins identified by CD antigens have been cloned and verified by standard molecular biology techniques. CD antigens have been characterized as both transmembrane proteins and cell surface proteins anchored to the plasma membrane via covalent attachment to fatty acid-containing glycolipids such as glycosylphosphatidylinositol (GPI). (Reviewed in Barclay, A.N. et al. (1995) The Leucocyte Antigen Facts Book, Academic Press, San Diego CA, pp. 17-20.)

#### Ion Channels

Ion channels are found in the plasma membranes of virtually every cell in the body. For example, chloride channels mediate a variety of cellular functions including regulation of membrane potentials and absorption and secretion of ions across epithelial membranes. Chloride channels also regulate the pH of organelles such as the Golgi apparatus and endosomes (see, e.g., Greger, R. (1988) *Annu. Rev. Physiol.* 50:111-122). Electrophysiological and pharmacological properties of chloride channels, including ion conductance, current-voltage relationships, and sensitivity to modulators, suggest that different chloride channels exist in muscles, neurons, fibroblasts, epithelial cells, and lymphocytes.

Many ion channels have sites for phosphorylation by one or more protein kinases including protein kinase A, protein kinase C, tyrosine kinase, and casein kinase II, all of which regulate ion channel activity in cells. Inappropriate phosphorylation of proteins in cells has been linked to changes in cell cycle progression and cell differentiation. Changes in the cell cycle have been linked to induction of apoptosis or cancer. Changes in cell differentiation have been linked to diseases and disorders of the reproductive system, immune system, skeletal muscle, and other organ systems.

#### Proton Pumps

Proton ATPases comprise a large class of membrane proteins that use the energy of ATP

hydrolysis to generate an electrochemical proton gradient across a membrane. The resultant gradient may be used to transport other ions across the membrane ( $\text{Na}^+$ ,  $\text{K}^+$ , or  $\text{Cl}^-$ ) or to maintain organelle pH. Proton ATPases are further subdivided into the mitochondrial F-ATPases, the plasma membrane ATPases, and the vacuolar ATPases. The vacuolar ATPases establish and maintain an acidic pH within various organelles involved in the processes of endocytosis and exocytosis (Mellman, I. et al. (1986) Annu. Rev. Biochem. 55:663-700).

Proton-coupled, 12 membrane-spanning domain transporters such as PEPT 1 and PEPT 2 are responsible for gastrointestinal absorption and for renal reabsorption of peptides using an electrochemical  $\text{H}^+$  gradient as the driving force. Another type of peptide transporter, the TAP transporter, is a heterodimer consisting of TAP 1 and TAP 2 and is associated with antigen processing. Peptide antigens are transported across the membrane of the endoplasmic reticulum by TAP so they can be expressed on the cell surface in association with MHC molecules. Each TAP protein consists of multiple hydrophobic membrane spanning segments and a highly conserved ATP-binding cassette (Boll, M. et al. (1996) Proc. Natl. Acad. Sci. USA 93:284-289). Pathogenic microorganisms, such as herpes simplex virus, may encode inhibitors of TAP-mediated peptide transport in order to evade immune surveillance (Marusina, K. and J.J. Manaco (1996) Curr. Opin. Hematol. 3:19-26).

#### ABC Transporters

The ATP-binding cassette (ABC) transporters, also called the "traffic ATPases", comprise a superfamily of membrane proteins that mediate transport and channel functions in prokaryotes and eukaryotes (Higgins, C.F. (1992) Annu. Rev. Cell Biol. 8:67-113). ABC proteins share a similar overall structure and significant sequence homology. All ABC proteins contain a conserved domain of approximately two hundred amino acid residues which includes one or more nucleotide binding domains. Mutations in ABC transporter genes are associated with various disorders, such as hyperbilirubinemia II/Dubin-Johnson syndrome, recessive Stargardt's disease, X-linked adrenoleukodystrophy, multidrug resistance, celiac disease, and cystic fibrosis.

#### Peripheral and Anchored Membrane Proteins

Some membrane proteins are not membrane-spanning but are attached to the plasma membrane via membrane anchors or interactions with integral membrane proteins. Membrane anchors are covalently joined to a protein post-translationally and include such moieties as prenyl, myristyl, and glycosylphosphatidyl inositol groups. Membrane localization of peripheral and anchored proteins is important for their function in processes such as receptor-mediated signal transduction. For example, prenylation of Ras is required for its localization to the plasma membrane and for its normal and oncogenic functions in signal transduction.

#### Vesicle Coat Proteins

Intercellular communication is essential for the development and survival of multicellular organisms. Cells communicate with one another through the secretion and uptake of protein signaling molecules. The uptake of proteins into the cell is achieved by the endocytic pathway, in which the interaction of extracellular signaling molecules with plasma membrane receptors results in the formation of plasma membrane-derived vesicles that enclose and transport the molecules into the cytosol. These transport vesicles fuse with and mature into endosomal and lysosomal (digestive) compartments. The secretion of proteins from the cell is achieved by exocytosis, in which molecules inside of the cell proceed through the secretory pathway. In this pathway, molecules transit from the ER to the Golgi apparatus and finally to the plasma membrane, where they are secreted from the cell.

Several steps in the transit of material along the secretory and endocytic pathways require the formation of transport vesicles. Specifically, vesicles form at the transitional endoplasmic reticulum (tER), the rim of Golgi cisternae, the face of the Trans-Golgi Network (TGN), the plasma membrane (PM), and tubular extensions of the endosomes. Vesicle formation occurs when a region of membrane buds off from the donor organelle. The membrane-bound vesicle contains proteins to be transported and is surrounded by a proteinaceous coat, the components of which are recruited from the cytosol. Two different classes of coat protein have been identified. Clathrin coats form on vesicles derived from the TGN and PM, whereas coatamer (COP) coats form on vesicles derived from the ER and Golgi. COP coats can be further classified as COPI, involved in retrograde traffic through the Golgi and from the Golgi to the ER, and COPII, involved in anterograde traffic from the ER to the Golgi (Mellman, *supra*).

In clathrin-based vesicle formation, adapter proteins bring vesicle cargo and coat proteins together at the surface of the budding membrane. Adapter protein-1 and -2 select cargo from the TGN and plasma membrane, respectively, based on molecular information encoded on the cytoplasmic tail of integral membrane cargo proteins. Adapter proteins also recruit clathrin to the budding site. Clathrin is a protein complex consisting of three large and three small polypeptide chains arranged in a three-legged structure called a triskelion. Multiple triskelions and other coat proteins appear to self-assemble on the membrane to form a coated pit. This assembly process may serve to deform the membrane into a budding vesicle. GTP-bound ADP-ribosylation factor (Arf) is also incorporated into the coated assembly. Another small G-protein, dynamin, forms a ring complex around the neck of the forming vesicle and may provide the mechanochemical force to seal the bud, thereby releasing the vesicle. The coated vesicle complex is then transported through the cytosol. During the transport process, Arf-bound GTP is hydrolyzed to GDP, and the coat dissociates from the transport vesicle (West, M.A. et al. (1997) *J. Cell Biol.* 138:1239-1254).

Vesicles which bud from the ER and the Golgi are covered with a protein coat similar to the clathrin coat of endocytic and TGN vesicles. The coat protein (COP) is assembled from cytosolic

precursor molecules at specific budding regions on the organelle. The COP coat consists of two major components, a G-protein (Arf or Sar) and coat protomer (coatamer). Coatamer is an equimolar complex of seven proteins, termed alpha-, beta-, beta'-, gamma-, delta-, epsilon- and zeta-COP. The coatamer complex binds to dilysine motifs contained on the cytoplasmic tails of integral membrane proteins. These include the KKXX retrieval motif of membrane proteins of the ER and dibasic/diphenylamine motifs of members of the p24 family. The p24 family of type I membrane proteins represent the major membrane proteins of COPI vesicles (Harter, C. and F.T. Wieland (1998) Proc. Natl. Acad. Sci. USA 95:11649-11654).

#### 10 Organelle Associated Molecules

SEQ ID NO:44, SEQ ID NO:45, and SEQ ID NO:46 encode, for example, organelle associated molecules.

Eukaryotic cells are organized into various cellular organelles which has the effect of separating specific molecules and their functions from one another and from the cytosol. Within the cell, various membrane structures surround and define these organelles while allowing them to interact with one another and the cell environment through both active and passive transport processes. Important cell organelles include the nucleus, the Golgi apparatus, the endoplasmic reticulum, mitochondria, peroxisomes, lysosomes, endosomes, and secretory vesicles.

#### Nucleus

The cell nucleus contains all of the genetic information of the cell in the form of DNA, and the components and machinery necessary for replication of DNA and for transcription of DNA into RNA. (See Alberts, B. et al. (1994) Molecular Biology of the Cell, Garland Publishing Inc., New York NY, pp. 335-399.) DNA is organized into compact structures in the nucleus by interactions with various DNA-binding proteins such as histones and non-histone chromosomal proteins.

DNA-specific nucleases, DNAses, partially degrade these compacted structures prior to DNA replication or transcription. DNA replication takes place with the aid of DNA helicases which unwind the double-stranded DNA helix, and DNA polymerases that duplicate the separated DNA strands.

Transcriptional regulatory proteins are essential for the control of gene expression. Some of these proteins function as transcription factors that initiate, activate, repress, or terminate gene transcription. Transcription factors generally bind to the promoter, enhancer, and upstream regulatory regions of a gene in a sequence-specific manner, although some factors bind regulatory elements within or downstream of a gene's coding region. Transcription factors may bind to a specific region of DNA singly or as a complex with other accessory factors. (Reviewed in Lewin, B. (1990) Genes IV, Oxford University Press, New York NY, and Cell Press, Cambridge MA, pp. 554-570.)

Many transcription factors incorporate DNA-binding structural motifs which comprise either  $\alpha$  helices or  $\beta$  sheets that bind to the major groove of DNA. Four well-characterized structural motifs are helix-turn-helix, zinc finger, leucine zipper, and helix-loop-helix. Proteins containing these motifs may act alone as monomers, or they may form homo- or heterodimers that interact with DNA.

Many neoplastic disorders in humans can be attributed to inappropriate gene expression. Malignant cell growth may result from either excessive expression of tumor promoting genes or insufficient expression of tumor suppressor genes (Cleary, M.L. (1992) Cancer Surv. 15:89-104). Chromosomal translocations may also produce chimeric loci which fuse the coding sequence of one gene with the regulatory regions of a second unrelated gene. Such an arrangement likely results in inappropriate gene transcription, potentially contributing to malignancy.

In addition, the immune system responds to infection or trauma by activating a cascade of events that coordinate the progressive selection, amplification, and mobilization of cellular defense mechanisms. A complex and balanced program of gene activation and repression is involved in this process. However, hyperactivity of the immune system as a result of improper or insufficient regulation of gene expression may result in considerable tissue or organ damage. This damage is well documented in immunological responses associated with arthritis, allergens, heart attack, stroke, and infections (Isselbacher, K.J. et al. (1996) Harrison's Principles of Internal Medicine, 13/e, McGraw Hill, Inc. and Teton Data Systems Software).

Transcription of DNA into RNA also takes place in the nucleus catalyzed by RNA polymerases. Three types of RNA polymerase exist. RNA polymerase I makes large ribosomal RNAs, while RNA polymerase III makes a variety of small, stable RNAs including 5S ribosomal RNA and the transfer RNAs (tRNA). RNA polymerase II transcribes genes that will be translated into proteins. The primary transcript of RNA polymerase II is called heterogenous nuclear RNA (hnRNA), and must be further processed by splicing to remove non-coding sequences called introns. RNA splicing is mediated by small nuclear ribonucleoprotein complexes, or snRNPs, producing mature messenger RNA (mRNA) which is then transported out of the nucleus for translation into proteins.

#### Nucleolus

The nucleolus is a highly organized subcompartment in the nucleus that contains high concentrations of RNA and proteins and functions mainly in ribosomal RNA synthesis and assembly (Alberts, et al. supra, pp. 379-382). Ribosomal RNA (rRNA) is a structural RNA that is complexed with proteins to form ribonucleoprotein structures called ribosomes. Ribosomes provide the platform on which protein synthesis takes place.

Ribosomes are assembled in the nucleolus initially from a large, 45S rRNA combined with a variety of proteins imported from the cytoplasm, as well as smaller, 5S rRNAs. Later processing of

the immature ribosome results in formation of smaller ribosomal subunits which are transported from the nucleolus to the cytoplasm where they are assembled into functional ribosomes.

### Endoplasmic Reticulum

In eukaryotes, proteins are synthesized within the endoplasmic reticulum (ER), delivered from the ER to the Golgi apparatus for post-translational processing and sorting, and transported from the Golgi to specific intracellular and extracellular destinations. Synthesis of integral membrane proteins, secreted proteins, and proteins destined for the lumen of a particular organelle occurs on the rough endoplasmic reticulum (ER). The rough ER is so named because of the rough appearance in electron micrographs imparted by the attached ribosomes on which protein synthesis proceeds. Synthesis of proteins destined for the ER actually begins in the cytosol with the synthesis of a specific signal peptide which directs the growing polypeptide and its attached ribosome to the ER membrane where the signal peptide is removed and protein synthesis is completed. Soluble proteins destined for the ER lumen, for secretion, or for transport to the lumen of other organelles pass completely into the ER lumen. Transmembrane proteins destined for the ER or for other cell membranes are translocated across the ER membrane but remain anchored in the lipid bilayer of the membrane by one or more membrane-spanning  $\alpha$ -helical regions.

Translocated polypeptide chains destined for other organelles or for secretion also fold and assemble in the ER lumen with the aid of certain "resident" ER proteins. Protein folding in the ER is aided by two principal types of protein isomerases, protein disulfide isomerase (PDI), and peptidyl-prolyl isomerase (PPI). PDI catalyzes the oxidation of free sulfhydryl groups in cysteine residues to form intramolecular disulfide bonds in proteins. PPI, an enzyme that catalyzes the isomerization of certain proline imide bonds in oligopeptides and proteins, is considered to govern one of the rate limiting steps in the folding of many proteins to their final functional conformation. The cyclophilins represent a major class of PPI that was originally identified as the major receptor for the immunosuppressive drug cyclosporin A (Handschumacher, R.E. et al. (1984) Science 226:544-547). Molecular "chaperones" such as BiP (binding protein) in the ER recognize incorrectly folded proteins as well as proteins not yet folded into their final form and bind to them, both to prevent improper aggregation between them, and to promote proper folding.

The "N-linked" glycosylation of most soluble secreted and membrane-bound proteins by oligosaccharides linked to asparagine residues in proteins is also performed in the ER. This reaction is catalyzed by a membrane-bound enzyme, oligosaccharyl transferase.

### Golgi Apparatus

The Golgi apparatus is a complex structure that lies adjacent to the ER in eukaryotic cells and serves primarily as a sorting and dispatching station for products of the ER (Alberts, et al. *supra*, pp. 600-610). Additional posttranslational processing, principally additional glycosylation, also occurs in

the Golgi. Indeed, the Golgi is a major site of carbohydrate synthesis, including most of the glycosaminoglycans of the extracellular matrix. N-linked oligosaccharides, added to proteins in the ER, are also further modified in the Golgi by the addition of more sugar residues to form complex N-linked oligosaccharides. "O-linked" glycosylation of proteins also occurs in the Golgi by addition of N-acetylgalactosamine to the hydroxyl group of a serine or threonine residue followed by the sequential addition of other sugar residues to the first. This process is catalyzed by a series of glycosyltransferases each specific for a particular donor sugar nucleotide and acceptor molecule (Lodish, H. et al. (1995) Molecular Cell Biology, W.H. Freeman and Co., New York NY, pp.700-708). In many cases, both N- and O-linked oligosaccharides appear to be required for the secretion of proteins or the movement of plasma membrane glycoproteins to the cell surface.

The terminal compartment of the Golgi is the Trans-Golgi Network (TGN), where both membrane and luminal proteins are sorted for their final destination. Transport (or secretory) vesicles destined for intracellular compartments, such as lysosomes, bud off of the TGN. Other transport vesicles bud off containing proteins destined for the plasma membrane, such as receptors, adhesion molecules, and ion channels, and secretory proteins, such as hormones, neurotransmitters, and digestive enzymes.

#### Vacuoles

The vacuole system is a collection of membrane bound compartments in eukaryotic cells that functions in the processes of endocytosis and exocytosis. They include phagosomes, lysosomes, endosomes, and secretory vesicles. Endocytosis is the process in cells of internalizing nutrients, solutes or small particles (pinocytosis) or large particles such as internalized receptors, viruses, bacteria, or bacterial toxins (phagocytosis). Exocytosis is the process of transporting molecules to the cell surface. It facilitates placement or localization of membrane-bound receptors or other membrane proteins and secretion of hormones, neurotransmitters, digestive enzymes, wastes, etc.

A common property of all of these vacuoles is an acidic pH environment ranging from approximately pH 4.5-5.0. This acidity is maintained by the presence of a proton ATPase that uses the energy of ATP hydrolysis to generate an electrochemical proton gradient across a membrane (Mellman, I. et al. (1986) *Annu. Rev. Biochem.* 55:663-700). Eukaryotic vacuolar proton ATPase (vp-ATPase) is a multimeric enzyme composed of 3-10 different subunits. One of these subunits is a highly hydrophobic polypeptide of approximately 16 kDa that is similar to the proteolipid component of vp-ATPases from eubacteria, fungi, and plant vacuoles (Mandel, M. et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:5521-5524). The 16 kDa proteolipid component is the major subunit of the membrane portion of vp-ATPase and functions in the transport of protons across the membrane.

#### Lysosomes

Lysosomes are membranous vesicles containing various hydrolytic enzymes used for the

controlled intracellular digestion of macromolecules. Lysosomes contain some 40 types of enzymes including proteases, nucleases, glycosidases, lipases, phospholipases, phosphatases, and sulfatases, all of which are acid hydrolases that function at a pH of about 5. Lysosomes are surrounded by a unique membrane containing transport proteins that allow the final products of macromolecule degradation, such as sugars, amino acids, and nucleotides, to be transported to the cytosol where they may be either excreted or reutilized by the cell. A vp-ATPase, such as that described above, maintains the acidic environment necessary for hydrolytic activity (Alberts, supra, pp. 610-611).

#### Endosomes

Endosomes are another type of acidic vacuole that is used to transport substances from the cell surface to the interior of the cell in the process of endocytosis. Like lysosomes, endosomes have an acidic environment provided by a vp-ATPase (Alberts et al. supra, pp. 610-618). Two types of endosomes are apparent based on tracer uptake studies that distinguish their time of formation in the cell and their cellular location. Early endosomes are found near the plasma membrane and appear to function primarily in the recycling of internalized receptors back to the cell surface. Late endosomes appear later in the endocytic process close to the Golgi apparatus and the nucleus, and appear to be associated with delivery of endocytosed material to lysosomes or to the TGN where they may be recycled. Specific proteins are associated with particular transport vesicles and their target compartments that may provide selectivity in targeting vesicles to their proper compartments. A cytosolic prenylated GTP-binding protein, Rab, is one such protein. Rabs 4, 5, and 11 are associated with the early endosome, whereas Rabs 7 and 9 associate with the late endosome.

#### Mitochondria

Mitochondria are oval-shaped organelles comprising an outer membrane, a tightly folded inner membrane, an intermembrane space between the outer and inner membranes, and a matrix inside the inner membrane. The outer membrane contains many porin molecules that allow ions and charged molecules to enter the intermembrane space, while the inner membrane contains a variety of transport proteins that transfer only selected molecules. Mitochondria are the primary sites of energy production in cells.

Energy is produced by the oxidation of glucose and fatty acids. Glucose is initially converted to pyruvate in the cytoplasm. Fatty acids and pyruvate are transported to the mitochondria for complete oxidation to CO<sub>2</sub> coupled by enzymes to the transport of electrons from NADH and FADH<sub>2</sub> to oxygen and to the synthesis of ATP (oxidative phosphorylation) from ADP and P<sub>i</sub>.

Pyruvate is transported into the mitochondria and converted to acetyl-CoA for oxidation via the citric acid cycle, involving pyruvate dehydrogenase components, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase. Enzymes involved in the citric acid cycle include: citrate synthetase, aconitases, isocitrate dehydrogenase, alpha-ketoglutarate dehydrogenase complex including



transsuccinylases, succinyl CoA synthetase, succinate dehydrogenase, fumarases, and malate dehydrogenase. Acetyl CoA is oxidized to CO<sub>2</sub> with concomitant formation of NADH, FADH<sub>2</sub>, and GTP. In oxidative phosphorylation, the transfer of electrons from NADH and FADH<sub>2</sub> to oxygen by dehydrogenases is coupled to the synthesis of ATP from ADP and P<sub>i</sub> by the F<sub>0</sub>F<sub>1</sub> ATPase complex in the mitochondrial inner membrane. Enzyme complexes responsible for electron transport and ATP synthesis include the F<sub>0</sub>F<sub>1</sub> ATPase complex, ubiquinone(CoQ)-cytochrome c reductase, ubiquinone reductase, cytochrome b, cytochrome c<sub>1</sub>, FeS protein, and cytochrome c oxidase.

#### Peroxisomes

Peroxisomes, like mitochondria, are a major site of oxygen utilization. They contain one or more enzymes, such as catalase and urate oxidase, that use molecular oxygen to remove hydrogen atoms from specific organic substrates in an oxidative reaction that produces hydrogen peroxide (Alberts, supra, pp. 574-577). Catalase oxidizes a variety of substrates including phenols, formic acid, formaldehyde, and alcohol and is important in peroxisomes of liver and kidney cells for detoxifying various toxic molecules that enter the bloodstream. Another major function of oxidative reactions in peroxisomes is the breakdown of fatty acids in a process called  $\beta$  oxidation.  $\beta$  oxidation results in shortening of the alkyl chain of fatty acids by blocks of two carbon atoms that are converted to acetyl CoA and exported to the cytosol for reuse in biosynthetic reactions.

Also like mitochondria, peroxisomes import their proteins from the cytosol using a specific signal sequence located near the C-terminus of the protein. The importance of this import process is evident in the inherited human disease Zellweger syndrome, in which a defect in importing proteins into peroxisomes leads to a peroxisomal deficiency resulting in severe abnormalities in the brain, liver, and kidneys, and death soon after birth. One form of this disease has been shown to be due to a mutation in the gene encoding a peroxisomal integral membrane protein called peroxisome assembly factor-1.

The discovery of new human molecules for diagnostics and therapeutics satisfies a need in the art by providing new compositions which are useful in the diagnosis, study, prevention, and treatment of diseases associated with human molecules.

#### **SUMMARY OF THE INVENTION**

The present invention relates to nucleic acid sequences comprising human diagnostic and therapeutic polynucleotides (dithp) as presented in the Sequence Listing. Some of the dithp uniquely identify genes encoding human structural, functional, and regulatory molecules.

The invention provides an isolated polynucleotide comprising a polynucleotide sequence selected from the group consisting of a) a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52; b) a naturally occurring polynucleotide sequence having at least 90% sequence

identity to a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52; c) a polynucleotide sequence complementary to a); d) a polynucleotide sequence complementary to b); and e) an RNA equivalent of a) through d). In one alternative, the polynucleotide comprises a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52. In another alternative, the polynucleotide comprises at least 60 contiguous nucleotides of a polynucleotide sequence selected from the group consisting of a) a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52; b) a naturally occurring polynucleotide sequence having at least 90% sequence identity to a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52; c) a polynucleotide sequence complementary to a); d) a polynucleotide sequence complementary to b); and e) an RNA equivalent of a) through d). The invention further provides a composition for the detection of expression of human diagnostic and therapeutic polynucleotides, comprising at least one isolated polynucleotide comprising a polynucleotide sequence selected from the group consisting of a) a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52; b) a naturally occurring polynucleotide sequence having at least 90% sequence identity to a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52; c) a polynucleotide sequence complementary to a); d) a polynucleotide sequence complementary to b); and e) an RNA equivalent of a) through d); and a detectable label.

The invention also provides a method for detecting a target polynucleotide in a sample, said target polynucleotide comprising a polynucleotide sequence selected from the group consisting of a) a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52; b) a naturally occurring polynucleotide sequence having at least 90% sequence identity to a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52; c) a polynucleotide sequence complementary to a); d) a polynucleotide sequence complementary to b); and e) an RNA equivalent of a) through d). The method comprises a) hybridizing the sample with a probe comprising at least 20 contiguous nucleotides comprising a sequence complementary to said target polynucleotide in the sample, and which probe specifically hybridizes to said target polynucleotide, under conditions whereby a hybridization complex is formed between said probe and said target polynucleotide, and b) detecting the presence or absence of said hybridization complex, and, optionally, if present, the amount thereof. In one alternative, the probe comprises at least 30 contiguous nucleotides. In another alternative, the probe comprises at least 60 contiguous nucleotides.

The invention further provides a recombinant polynucleotide comprising a promoter sequence operably linked to an isolated polynucleotide comprising a polynucleotide sequence selected from the group consisting of a) a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52; b) a naturally occurring polynucleotide sequence having at least 90% sequence identity to a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52; c) a polynucleotide

sequence complementary to a); d) a polynucleotide sequence complementary to b); and e) an RNA equivalent of a) through d). In one alternative, the invention provides a cell transformed with the recombinant polynucleotide. In another alternative, the invention provides a transgenic organism comprising the recombinant polynucleotide. In a further alternative, the invention provides a method for producing a human diagnostic and therapeutic polypeptide, the method comprising a) culturing a cell under conditions suitable for expression of the human diagnostic and therapeutic polypeptide, wherein said cell is transformed with the recombinant polynucleotide, and b) recovering the human diagnostic and therapeutic polypeptide so expressed.

The invention also provides a purified human diagnostic and therapeutic polypeptide (DITHP) encoded by at least one polynucleotide comprising a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52. Additionally, the invention provides an isolated antibody which specifically binds to the human diagnostic and therapeutic polypeptide. The invention further provides a method of identifying a test compound which specifically binds to the human diagnostic and therapeutic polypeptide, the method comprising the steps of a) providing a test compound; b) combining the human diagnostic and therapeutic polypeptide with the test compound for a sufficient time and under suitable conditions for binding; and c) detecting binding of the human diagnostic and therapeutic polypeptide to the test compound, thereby identifying the test compound which specifically binds the human diagnostic and therapeutic polypeptide.

The invention further provides a microarray wherein at least one element of the microarray is an isolated polynucleotide comprising at least 60 contiguous nucleotides of a polynucleotide comprising a polynucleotide sequence selected from the group consisting of a) a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52; b) a naturally occurring polynucleotide sequence having at least 90% sequence identity to a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52; c) a polynucleotide sequence complementary to a); d) a polynucleotide sequence complementary to b); and e) an RNA equivalent of a) through d). The invention also provides a method for generating a transcript image of a sample which contains polynucleotides. The method comprises a) labeling the polynucleotides of the sample, b) contacting the elements of the microarray with the labeled polynucleotides of the sample under conditions suitable for the formation of a hybridization complex, and c) quantifying the expression of the polynucleotides in the sample.

Additionally, the invention provides a method for screening a compound for effectiveness in altering expression of a target polynucleotide, wherein said target polynucleotide comprises a polynucleotide sequence selected from the group consisting of a) a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52; b) a naturally occurring polynucleotide sequence having at least 90% sequence identity to a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52; c) a polynucleotide sequence complementary to a); d) a polynucleotide sequence

complementary to b); and e) an RNA equivalent of a) through d). The method comprises a) exposing a sample comprising the target polynucleotide to a compound, and b) detecting altered expression of the target polynucleotide.

The invention further provides a method for detecting a target polynucleotide in a sample for toxicity testing of a compound, said target polynucleotide comprising a polynucleotide sequence selected from the group consisting of a) a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52; b) a naturally occurring polynucleotide sequence having at least 90% sequence identity to a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52; c) a polynucleotide sequence complementary to a); d) a polynucleotide sequence complementary to b); and c) an RNA equivalent of a) through d). The method comprises a) hybridizing the sample with a probe comprising at least 20 contiguous nucleotides comprising a sequence complementary to said target polynucleotide in the sample, and which probe specifically hybridizes to said target polynucleotide, under conditions whereby a hybridization complex is formed between said probe and said target polynucleotide; b) detecting the presence or absence of said hybridization complex, and, optionally, if present, the amount thereof, and c) comparing the presence, absence or amount of said target polynucleotide in a first biological sample and a second biological sample, wherein said first biological sample has been contacted with said compound, and said second sample is a control, whereby a change in presence, absence or amount of said target polynucleotide in said first sample, as compared with said second sample, is indicative of toxic response to said compound.

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### DESCRIPTION OF THE TABLES

Table 1 shows the sequence identification numbers (SEQ ID NO:s) and template identification numbers (template IDs) corresponding to the polynucleotides of the present invention, along with their GenBank hits (GI Numbers), probability scores, and functional annotations corresponding to the GenBank hits.

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Table 2 shows the sequence identification numbers (SEQ ID NO:s) and template identification numbers (template IDs) corresponding to the polynucleotides of the present invention, along with polynucleotide segments of each template sequence as defined by the indicated "start" and "stop" nucleotide positions. The reading frames of the polynucleotide segments and the Pfam hits, Pfam descriptions, and E-values corresponding to the polypeptide domains encoded by the polynucleotide segments are indicated.

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Table 3 shows the sequence identification numbers (SEQ ID NO:s) and template identification numbers (template IDs) corresponding to the polynucleotides of the present invention, along with polynucleotide segments of each template sequence as defined by the indicated "start" and "stop" nucleotide positions. The reading frames of the polynucleotide segments are shown, and the

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polypeptides encoded by the polynucleotide segments constitute either signal peptide (SP) or transmembrane (TM) domains, as indicated.

Table 4 shows the sequence identification numbers (SEQ ID NO:s) and template identification numbers (template IDs) corresponding to the polynucleotides of the present invention, along with component sequence identification numbers (component IDs) corresponding to each template. The component sequences, which were used to assemble the template sequences, are defined by the indicated "start" and "stop" nucleotide positions along each template.

Table 5 summarizes the bioinformatics tools which are useful for analysis of the polynucleotides of the present invention. The first column of Table 5 lists analytical tools, programs, and algorithms, the second column provides brief descriptions thereof, the third column presents appropriate references, all of which are incorporated by reference herein in their entirety, and the fourth column presents, where applicable, the scores, probability values, and other parameters used to evaluate the strength of a match between two sequences (the higher the score, the greater the homology between two sequences).

## DETAILED DESCRIPTION OF THE INVENTION

Before the nucleic acid sequences and methods are presented, it is to be understood that this invention is not limited to the particular machines, methods, and materials described. Although particular embodiments are described, machines, methods, and materials similar or equivalent to these embodiments may be used to practice the invention. The preferred machines, methods, and materials set forth are not intended to limit the scope of the invention which is limited only by the appended claims.

The singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. All technical and scientific terms have the meanings commonly understood by one of ordinary skill in the art. All publications are incorporated by reference for the purpose of describing and disclosing the cell lines, vectors, and methodologies which are presented and which might be used in connection with the invention. Nothing in the specification is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

### Definitions

As used herein, the lower case "dithp" refers to a nucleic acid sequence, while the upper case "DITHP" refers to an amino acid sequence encoded by dithp. A "full-length" dithp refers to a nucleic acid sequence containing the entire coding region of a gene endogenously expressed in human tissue.

"Adjuvants" are materials such as Freund's adjuvant, mineral gels (aluminum hydroxide), and surface active substances (lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole

limpet hemocyanin, and dinitrophenol) which may be administered to increase a host's immunological response.

"Allele" refers to an alternative form of a nucleic acid sequence. Alleles result from a "mutation," a change or an alternative reading of the genetic code. Any given gene may have none, one, or many allelic forms. Mutations which give rise to alleles include deletions, additions, or substitutions of nucleotides. Each of these changes may occur alone, or in combination with the others, one or more times in a given nucleic acid sequence. The present invention encompasses allelic dithp.

"Amino acid sequence" refers to a peptide, a polypeptide, or a protein of either natural or synthetic origin. The amino acid sequence is not limited to the complete, endogenous amino acid sequence and may be a fragment, epitope, variant, or derivative of a protein expressed by a nucleic acid sequence.

"Amplification" refers to the production of additional copies of a sequence and is carried out using polymerase chain reaction (PCR) technologies well known in the art.

"Antibody" refers to intact molecules as well as to fragments thereof, such as Fab, F(ab')<sub>2</sub>, and Fv fragments, which are capable of binding the epitopic determinant. Antibodies that bind DITHP polypeptides can be prepared using intact polypeptides or using fragments containing small peptides of interest as the immunizing antigen. The polypeptide or peptide used to immunize an animal (e.g., a mouse, a rat, or a rabbit) can be derived from the translation of RNA, or synthesized chemically, and can be conjugated to a carrier protein if desired. Commonly used carriers that are chemically coupled to peptides include bovine serum albumin, thyroglobulin, and keyhole limpet hemocyanin (KLH). The coupled peptide is then used to immunize the animal.

"Antisense sequence" refers to a sequence capable of specifically hybridizing to a target sequence. The antisense sequence may include DNA, RNA, or any nucleic acid mimic or analog such as peptide nucleic acid (PNA); oligonucleotides having modified backbone linkages such as phosphorothioates, methylphosphonates, or benzylphosphonates; oligonucleotides having modified sugar groups such as 2'-methoxyethyl sugars or 2'-methoxyethoxy sugars; or oligonucleotides having modified bases such as 5-methyl cytosine, 2'-deoxyuracil, or 7-deaza-2'-deoxyguanosine.

"Antisense sequence" refers to a sequence capable of specifically hybridizing to a target sequence. The antisense sequence can be DNA, RNA, or any nucleic acid mimic or analog.

"Antisense technology" refers to any technology which relies on the specific hybridization of an antisense sequence to a target sequence.

A "bin" is a portion of computer memory space used by a computer program for storage of data, and bounded in such a manner that data stored in a bin may be retrieved by the program.

"Biologically active" refers to an amino acid sequence having a structural, regulatory, or biochemical function of a naturally occurring amino acid sequence.

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"Clone joining" is a process for combining gene bins based upon the bins' containing sequence information from the same clone. The sequences may assemble into a primary gene transcript as well as one or more splice variants.

"Complementary" describes the relationship between two single-stranded nucleic acid sequences that anneal by base-pairing (5'-A-G-T-3' pairs with its complement 3'-T-C-A-5').

A "component sequence" is a nucleic acid sequence selected by a computer program such as PHRED and used to assemble a consensus or template sequence from one or more component sequences.

A "consensus sequence" or "template sequence" is a nucleic acid sequence which has been assembled from overlapping sequences, using a computer program for fragment assembly such as the GELVIEW fragment assembly system (Genetics Computer Group (GCG), Madison WI) or using a relational database management system (RDMS).

"Conservative amino acid substitutions" are those substitutions that, when made, least interfere with the properties of the original protein, i.e., the structure and especially the function of the protein is conserved and not significantly changed by such substitutions. The table below shows amino acids which may be substituted for an original amino acid in a protein and which are regarded as conservative substitutions.

	Original Residue	Conservative Substitution
20	Ala	Gly, Ser
	Arg	His, Lys
	Asn	Asp, Gln, His
	Asp	Asn, Glu
	Cys	Ala, Ser
25	Gln	Asn, Glu, His
	Glu	Asp, Gln, His
	Gly	Ala
	His	Asn, Arg, Gln, Glu
	Ile	Leu, Val
30	Leu	Ile, Val
	Lys	Arg, Gln, Glu
	Met	Leu, Ile
	Phe	His, Met, Leu, Trp, Tyr
	Ser	Cys, Thr
35	Thr	Ser, Val
	Trp	Phe, Tyr
	Tyr	His, Phe, Trp
	Val	Ile, Leu, Thr

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Conservative substitutions generally maintain (a) the structure of the polypeptide backbone in

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the area of the substitution, for example, as a beta sheet or alpha helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain.

"Deletion" refers to a change in either a nucleic or amino acid sequence in which at least one nucleotide or amino acid residue, respectively, is absent.

- 5 "Derivative" refers to the chemical modification of a nucleic acid sequence, such as by replacement of hydrogen by an alkyl, acyl, amino, hydroxyl, or other group.

"E-value" refers to the statistical probability that a match between two sequences occurred by chance.

- A "fragment" is a unique portion of dithp or DITHP which is identical in sequence to but  
10 shorter in length than the parent sequence. A fragment may comprise up to the entire length of the defined sequence, minus one nucleotide/amino acid residue. For example, a fragment may comprise from 10 to 1000 contiguous amino acid residues or nucleotides. A fragment used as a probe, primer, antigen, therapeutic molecule, or for other purposes, may be at least 5, 10, 15, 16, 20, 25, 30, 40, 50, 60, 75, 100, 150, 250 or at least 500 contiguous amino acid residues or nucleotides in length.

- 15 Fragments may be preferentially selected from certain regions of a molecule. For example, a polypeptide fragment may comprise a certain length of contiguous amino acids selected from the first 250 or 500 amino acids (or first 25% or 50%) of a polypeptide as shown in a certain defined sequence. Clearly these lengths are exemplary, and any length that is supported by the specification, including the Sequence Listing and the figures, may be encompassed by the present embodiments.

- 20 A fragment of dithp comprises a region of unique polynucleotide sequence that specifically identifies dithp, for example, as distinct from any other sequence in the same genome. A fragment of dithp is useful, for example, in hybridization and amplification technologies and in analogous methods that distinguish dithp from related polynucleotide sequences. The precise length of a fragment of dithp and the region of dithp to which the fragment corresponds are routinely determinable by one of ordinary  
25 skill in the art based on the intended purpose for the fragment.

- A fragment of DITHP is encoded by a fragment of dithp. A fragment of DITHP comprises a region of unique amino acid sequence that specifically identifies DITHP. For example, a fragment of DITHP is useful as an immunogenic peptide for the development of antibodies that specifically recognize DITHP. The precise length of a fragment of DITHP and the region of DITHP to which the  
30 fragment corresponds are routinely determinable by one of ordinary skill in the art based on the intended purpose for the fragment.

A "full length" nucleotide sequence is one containing at least a start site for translation to a protein sequence, followed by an open reading frame and a stop site, and encoding a "full length" polypeptide.

- 35 "Hit" refers to a sequence whose annotation will be used to describe a given template. Criteria



for selecting the top hit are as follows: if the template has one or more exact nucleic acid matches, the top hit is the exact match with highest percent identity. If the template has no exact matches but has significant protein hits, the top hit is the protein hit with the lowest E-value. If the template has no significant protein hits, but does have significant non-exact nucleotide hits, the top hit is the nucleotide hit with the lowest E-value.

"Homology" refers to sequence similarity either between a reference nucleic acid sequence and at least a fragment of a dithp or between a reference amino acid sequence and a fragment of a DITHP.

"Hybridization" refers to the process by which a strand of nucleotides anneals with a complementary strand through base pairing. Specific hybridization is an indication that two nucleic acid sequences share a high degree of identity. Specific hybridization complexes form under defined annealing conditions, and remain hybridized after the "washing" step. The defined hybridization conditions include the annealing conditions and the washing step(s), the latter of which is particularly important in determining the stringency of the hybridization process, with more stringent conditions allowing less non-specific binding, i.e., binding between pairs of nucleic acid probes that are not perfectly matched. Permissive conditions for annealing of nucleic acid sequences are routinely determinable and may be consistent among hybridization experiments, whereas wash conditions may be varied among experiments to achieve the desired stringency.

Generally, stringency of hybridization is expressed with reference to the temperature under which the wash step is carried out. Generally, such wash temperatures are selected to be about 5°C to 20°C lower than the thermal melting point ( $T_m$ ) for the specific sequence at a defined ionic strength and pH. The  $T_m$  is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. An equation for calculating  $T_m$  and conditions for nucleic acid hybridization is well known and can be found in Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup> ed., vol. 1-3, Cold Spring Harbor Press, Plainville NY; specifically see volume 2, chapter 9.

High stringency conditions for hybridization between polynucleotides of the present invention include wash conditions of 68°C in the presence of about 0.2 x SSC and about 0.1% SDS, for 1 hour. Alternatively, temperatures of about 65°C, 60°C, or 55°C may be used. SSC concentration may be varied from about 0.2 to 2 x SSC, with SDS being present at about 0.1%. Typically, blocking reagents are used to block non-specific hybridization. Such blocking reagents include, for instance, denatured salmon sperm DNA at about 100-200 µg/ml. Useful variations on these conditions will be readily apparent to those skilled in the art. Hybridization, particularly under high stringency conditions, may be suggestive of evolutionary similarity between the nucleotides. Such similarity is strongly indicative of a similar role for the nucleotides and their resultant proteins.

Other parameters, such as temperature, salt concentration, and detergent concentration may be

varied to achieve the desired stringency. Denaturants, such as formamide at a concentration of about 35-50% v/v, may also be used under particular circumstances, such as RNA:DNA hybridizations. Appropriate hybridization conditions are routinely determinable by one of ordinary skill in the art.

“Immunogenic” describes the potential for a natural, recombinant, or synthetic peptide, epitope, polypeptide, or protein to induce antibody production in appropriate animals, cells, or cell lines.

“Insertion” or “addition” refers to a change in either a nucleic or amino acid sequence in which at least one nucleotide or residue, respectively, is added to the sequence.

“Labeling” refers to the covalent or noncovalent joining of a polynucleotide, polypeptide, or antibody with a reporter molecule capable of producing a detectable or measurable signal.

“Microarray” is any arrangement of nucleic acids, amino acids, antibodies, etc., on a substrate. The substrate may be a solid support such as beads, glass, paper, nitrocellulose, nylon, or an appropriate membrane.

The terms “element” and “array element” refer to a polynucleotide, polypeptide, or other chemical compound having a unique and defined position on a microarray.

“Linkers” are short stretches of nucleotide sequence which may be added to a vector or a dithp to create restriction endonuclease sites to facilitate cloning. “Polylinkers” are engineered to incorporate multiple restriction enzyme sites and to provide for the use of enzymes which leave 5' or 3' overhangs (e.g., BamHI, EcoRI, and HindIII) and those which provide blunt ends (e.g., EcoRV, SnaBI, and StuI).

“Naturally occurring” refers to an endogenous polynucleotide or polypeptide that may be isolated from viruses or prokaryotic or eukaryotic cells.

“Nucleic acid sequence” refers to the specific order of nucleotides joined by phosphodiester bonds in a linear, polymeric arrangement. Depending on the number of nucleotides, the nucleic acid sequence can be considered an oligomer, oligonucleotide, or polynucleotide. The nucleic acid can be DNA, RNA, or any nucleic acid analog, such as PNA, may be of genomic or synthetic origin, may be either double-stranded or single-stranded, and can represent either the sense or antisense (complementary) strand.

“Oligomer” refers to a nucleic acid sequence of at least about 6 nucleotides and as many as about 60 nucleotides, preferably about 15 to 40 nucleotides, and most preferably between about 20 and 30 nucleotides, that may be used in hybridization or amplification technologies. Oligomers may be used as, e.g., primers for PCR, and are usually chemically synthesized.

“Operably linked” refers to the situation in which a first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences may be in close proximity or contiguous and, where necessary to join two protein coding regions, in the same reading frame.

"Peptide nucleic acid" (PNA) refers to a DNA mimic in which nucleotide bases are attached to a pseudopeptide backbone to increase stability. PNAs, also designated antigene agents, can prevent gene expression by targeting complementary messenger RNA.

The phrases "percent identity" and "% identity", as applied to polynucleotide sequences, refer to the percentage of residue matches between at least two polynucleotide sequences aligned using a standardized algorithm. Such an algorithm may insert, in a standardized and reproducible way, gaps in the sequences being compared in order to optimize alignment between two sequences, and therefore achieve a more meaningful comparison of the two sequences.

Percent identity between polynucleotide sequences may be determined using the default parameters of the CLUSTAL V algorithm as incorporated into the MEGALIGN version 3.12e sequence alignment program. This program is part of the LASERGENE software package, a suite of molecular biological analysis programs (DNASTAR, Madison WI). CLUSTAL V is described in Higgins, D.G. and Sharp, P.M. (1989) CABIOS 5:151-153 and in Higgins, D.G. et al. (1992) CABIOS 8:189-191. For pairwise alignments of polynucleotide sequences, the default parameters are set as follows: Ktuple=2, gap penalty=5, window=4, and "diagonals saved"=4. The "weighted" residue weight table is selected as the default. Percent identity is reported by CLUSTAL V as the "percent similarity" between aligned polynucleotide sequence pairs.

Alternatively, a suite of commonly used and freely available sequence comparison algorithms is provided by the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (Altschul, S.F. et al. (1990) J. Mol. Biol. 215:403-410), which is available from several sources, including the NCBI, Bethesda, MD, and on the Internet at <http://www.ncbi.nlm.nih.gov/BLAST/>. The BLAST software suite includes various sequence analysis programs including "blastn," that is used to determine alignment between a known polynucleotide sequence and other sequences on a variety of databases. Also available is a tool called "BLAST 2 Sequences" that is used for direct pairwise comparison of two nucleotide sequences. "BLAST 2 Sequences" can be accessed and used interactively at <http://www.ncbi.nlm.nih.gov/gorf/bl2/>. The "BLAST 2 Sequences" tool can be used for both blastn and blastp (discussed below). BLAST programs are commonly used with gap and other parameters set to default settings. For example, to compare two nucleotide sequences, one may use blastn with the "BLAST 2 Sequences" tool Version 2.0.9 (May-07-1999) set at default parameters. Such default parameters may be, for example:

*Matrix: BLOSUM62*  
*Reward for match: 1*  
*Penalty for mismatch: -2*  
*Open Gap: 5 and Extension Gap: 2 penalties*  
*Gap x drop-off: 50*

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*Expect: 10*

*Word Size: 11*

*Filter: on*

Percent identity may be measured over the length of an entire defined sequence, for example, as  
 5 defined by a particular SEQ ID number, or may be measured over a shorter length, for example, over  
 the length of a fragment taken from a larger, defined sequence, for instance, a fragment of at least 20, at  
 least 30, at least 40, at least 50, at least 70, at least 100, or at least 200 contiguous nucleotides. Such  
 lengths are exemplary only, and it is understood that any fragment length supported by the sequences  
 shown herein, in figures or Sequence Listings, may be used to describe a length over which percentage  
 10 identity may be measured.

Nucleic acid sequences that do not show a high degree of identity may nevertheless encode  
 similar amino acid sequences due to the degeneracy of the genetic code. It is understood that changes in  
 nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid sequences  
 that all encode substantially the same protein.

15 The phrases "percent identity" and "% identity", as applied to polypeptide sequences, refer to  
 the percentage of residue matches between at least two polypeptide sequences aligned using a  
 standardized algorithm. Methods of polypeptide sequence alignment are well-known. Some alignment  
 methods take into account conservative amino acid substitutions. Such conservative substitutions,  
 explained in more detail above, generally preserve the hydrophobicity and acidity of the substituted  
 20 residue, thus preserving the structure (and therefore function) of the folded polypeptide.

Percent identity between polypeptide sequences may be determined using the default parameters  
 of the CLUSTAL V algorithm as incorporated into the MEGALIGN version 3.12e sequence alignment  
 program (described and referenced above). For pairwise alignments of polypeptide sequences using  
 CLUSTAL V, the default parameters are set as follows: Ktuple=1, gap penalty=3, window=5, and  
 25 "diagonals saved"=5. The PAM250 matrix is selected as the default residue weight table. As with  
 polynucleotide alignments, the percent identity is reported by CLUSTAL V as the "percent similarity"  
 between aligned polypeptide sequence pairs.

Alternatively the NCBI BLAST software suite may be used. For example, for a pairwise  
 comparison of two polypeptide sequences, one may use the "BLAST 2 Sequences" tool Version 2.0.9  
 30 (May-07-1999) with blastp set at default parameters. Such default parameters may be, for example:

*Matrix: BLOSUM62*

*Open Gap: 11 and Extension Gap: 1 penalty*

*Gap x drop-off: 50*

*Expect: 10*

35 *Word Size: 3*

*Filter: on*

Percent identity may be measured over the length of an entire defined polypeptide sequence, for example, as defined by a particular SEQ ID number, or may be measured over a shorter length, for example, over the length of a fragment taken from a larger, defined polypeptide sequence, for instance, a fragment of at least 15, at least 20, at least 30, at least 40, at least 50, at least 70 or at least 150 contiguous residues. Such lengths are exemplary only, and it is understood that any fragment length supported by the sequences shown herein, in figures or Sequence Listings, may be used to describe a length over which percentage identity may be measured.

"Post-translational modification" of a DITHP may involve lipidation, glycosylation, phosphorylation, acetylation, racemization, proteolytic cleavage, and other modifications known in the art. These processes may occur synthetically or biochemically. Biochemical modifications will vary by cell type depending on the enzymatic milieu and the DITHP.

"Probe" refers to dithp or fragments thereof, which are used to detect identical, allelic or related nucleic acid sequences. Probes are isolated oligonucleotides or polynucleotides attached to a detectable label or reporter molecule. Typical labels include radioactive isotopes, ligands, chemiluminescent agents, and enzymes. "Primers" are short nucleic acids, usually DNA oligonucleotides, which may be annealed to a target polynucleotide by complementary base-pairing. The primer may then be extended along the target DNA strand by a DNA polymerase enzyme. Primer pairs can be used for amplification (and identification) of a nucleic acid sequence, e.g., by the polymerase chain reaction (PCR).

Probes and primers as used in the present invention typically comprise at least 15 contiguous nucleotides of a known sequence. In order to enhance specificity, longer probes and primers may also be employed, such as probes and primers that comprise at least 20, 30, 40, 50, 60, 70, 80, 90, 100, or at least 150 consecutive nucleotides of the disclosed nucleic acid sequences. Probes and primers may be considerably longer than these examples, and it is understood that any length supported by the specification, including the figures and Sequence Listing, may be used.

Methods for preparing and using probes and primers are described in the references, for example Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup> ed., vol. 1-3, Cold Spring Harbor Press, Plainview NY; Ausubel et al., 1987, Current Protocols in Molecular Biology, Greene Publ. Assoc. & Wiley-Intersciences, New York NY; Innis et al., 1990, PCR Protocols, A Guide to Methods and Applications, Academic Press, San Diego CA. PCR primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, 1991, Whitehead Institute for Biomedical Research, Cambridge MA).

Oligonucleotides for use as primers are selected using software known in the art for such purpose. For example, OLIGO 4.06 software is useful for the selection of PCR primer pairs of up to 100 nucleotides each, and for the analysis of oligonucleotides and larger polynucleotides of up to 5,000

nucleotides from an input polynucleotide sequence of up to 32 kilobases. Similar primer selection programs have incorporated additional features for expanded capabilities. For example, the PrimOU primer selection program (available to the public from the Genome Center at University of Texas South West Medical Center, Dallas TX) is capable of choosing specific primers from megabase sequences and is thus useful for designing primers on a genome-wide scope. The Primer3 primer selection program (available to the public from the Whitehead Institute/MIT Center for Genome Research, Cambridge MA) allows the user to input a "mispriming library," in which sequences to avoid as primer binding sites are user-specified. Primer3 is useful, in particular, for the selection of oligonucleotides for microarrays. (The source code for the latter two primer selection programs may also be obtained from their respective sources and modified to meet the user's specific needs.) The PrimeGen program (available to the public from the UK Human Genome Mapping Project Resource Centre, Cambridge UK) designs primers based on multiple sequence alignments, thereby allowing selection of primers that hybridize to either the most conserved or least conserved regions of aligned nucleic acid sequences. Hence, this program is useful for identification of both unique and conserved oligonucleotides and polynucleotide fragments. The oligonucleotides and polynucleotide fragments identified by any of the above selection methods are useful in hybridization technologies, for example, as PCR or sequencing primers, microarray elements, or specific probes to identify fully or partially complementary polynucleotides in a sample of nucleic acids. Methods of oligonucleotide selection are not limited to those described above.

"Purified" refers to molecules, either polynucleotides or polypeptides that are isolated or separated from their natural environment and are at least 60% free, preferably at least 75% free, and most preferably at least 90% free from other compounds with which they are naturally associated.

A "recombinant nucleic acid" is a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two or more otherwise separated segments of sequence.

This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques such as those described in Sambrook, supra. The term recombinant includes nucleic acids that have been altered solely by addition, substitution, or deletion of a portion of the nucleic acid. Frequently, a recombinant nucleic acid may include a nucleic acid sequence operably linked to a promoter sequence. Such a recombinant nucleic acid may be part of a vector that is used, for example, to transform a cell.

Alternatively, such recombinant nucleic acids may be part of a viral vector, e.g., based on a vaccinia virus, that could be used to vaccinate a mammal wherein the recombinant nucleic acid is expressed, inducing a protective immunological response in the mammal.

"Regulatory element" refers to a nucleic acid sequence from nontranslated regions of a gene, and includes enhancers, promoters, introns, and 3' untranslated regions, which interact with host

proteins to carry out or regulate transcription or translation.

"Reporter" molecules are chemical or biochemical moieties used for labeling a nucleic acid, an amino acid, or an antibody. They include radionuclides; enzymes; fluorescent, chemiluminescent, or chromogenic agents; substrates; cofactors; inhibitors; magnetic particles; and other moieties known in the art.

An "RNA equivalent," in reference to a DNA sequence, is composed of the same linear sequence of nucleotides as the reference DNA sequence with the exception that all occurrences of the nitrogenous base thymine are replaced with uracil, and the sugar backbone is composed of ribose instead of deoxyribose.

"Sample" is used in its broadest sense. Samples may contain nucleic acid or amino acids, antibodies, or other materials, and may be derived from any source (e.g., bodily fluids including, but not limited to, saliva, blood, and urine; chromosome(s), organelles, or membranes isolated from a cell; genomic DNA, RNA, or cDNA in solution or bound to a substrate; and cleared cells or tissues or blots or imprints from such cells or tissues).

"Specific binding" or "specifically binding" refers to the interaction between a protein or peptide and its agonist, antibody, antagonist, or other binding partner. The interaction is dependent upon the presence of a particular structure of the protein, e.g., the antigenic determinant or epitope, recognized by the binding molecule. For example, if an antibody is specific for epitope "A," the presence of a polypeptide containing epitope A, or the presence of free unlabeled A, in a reaction containing free labeled A and the antibody will reduce the amount of labeled A that binds to the antibody.

"Substitution" refers to the replacement of at least one nucleotide or amino acid by a different nucleotide or amino acid.

"Substrate" refers to any suitable rigid or semi-rigid support including, e.g., membranes, filters, chips, slides, wafers, fibers, magnetic or nonmagnetic beads, gels, tubing, plates, polymers, microparticles or capillaries. The substrate can have a variety of surface forms, such as wells, trenches, pins, channels and pores, to which polynucleotides or polypeptides are bound.

A "transcript image" refers to the collective pattern of gene expression by a particular tissue or cell type under given conditions at a given time.

"Transformation" refers to a process by which exogenous DNA enters a recipient cell. Transformation may occur under natural or artificial conditions using various methods well known in the art. Transformation may rely on any known method for the insertion of foreign nucleic acid sequences into a prokaryotic or eukaryotic host cell. The method is selected based on the host cell being transformed.

"Transformants" include stably transformed cells in which the inserted DNA is capable of

replication either as an autonomously replicating plasmid or as part of the host chromosome, as well as cells which transiently express inserted DNA or RNA.

A "transgenic organism," as used herein, is any organism, including but not limited to animals and plants, in which one or more of the cells of the organism contains heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques well known in the art. The nucleic acid is introduced into the cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or *in vitro* fertilization, but rather is directed to the introduction of a recombinant DNA molecule. The transgenic organisms contemplated in accordance with the present invention include bacteria, cyanobacteria, fungi, and plants and animals. The isolated DNA of the present invention can be introduced into the host by methods known in the art, for example infection, transfection, transformation or transconjugation. Techniques for transferring the DNA of the present invention into such organisms are widely known and provided in references such as Sambrook et al. (1989), supra.

A "variant" of a particular nucleic acid sequence is defined as a nucleic acid sequence having at least 25% sequence identity to the particular nucleic acid sequence over a certain length of one of the nucleic acid sequences using blastn with the "BLAST 2 Sequences" tool Version 2.0.9 (May-07-1999) set at default parameters. Such a pair of nucleic acids may show, for example, at least 30%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or even at least 98% or greater sequence identity over a certain defined length. The variant may result in "conservative" amino acid changes which do not affect structural and/or chemical properties. A variant may be described as, for example, an "allelic" (as defined above), "splice," "species," or "polymorphic" variant. A splice variant may have significant identity to a reference molecule, but will generally have a greater or lesser number of polynucleotides due to alternate splicing of exons during mRNA processing. The corresponding polypeptide may possess additional functional domains or lack domains that are present in the reference molecule. Species variants are polynucleotide sequences that vary from one species to another. The resulting polypeptides generally will have significant amino acid identity relative to each other. A polymorphic variant is a variation in the polynucleotide sequence of a particular gene between individuals of a given species. Polymorphic variants also may encompass "single nucleotide polymorphisms" (SNPs) in which the polynucleotide sequence varies by one base. The presence of SNPs may be indicative of, for example, a certain population, a disease state, or a propensity for a disease state.

In an alternative, variants of the polynucleotides of the present invention may be generated through recombinant methods. One possible method is a DNA shuffling technique such as MOLECULARBREEDING (Maxygen Inc., Santa Clara CA; described in U.S. Patent Number



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5,837,458; Chang, C.-C. et al. (1999) Nat. Biotechnol. 17:793-797; Christians, F.C. et al. (1999) Nat. Biotechnol. 17:259-264; and Cramer, A. et al. (1996) Nat. Biotechnol. 14:315-319) to alter or improve the biological properties of DITHP, such as its biological or enzymatic activity or its ability to bind to other molecules or compounds. DNA shuffling is a process by which a library of gene variants is produced using PCR-mediated recombination of gene fragments. The library is then subjected to selection or screening procedures that identify those gene variants with the desired properties. These preferred variants may then be pooled and further subjected to recursive rounds of DNA shuffling and selection/screening. Thus, genetic diversity is created through "artificial" breeding and rapid molecular evolution. For example, fragments of a single gene containing random point mutations may be recombined, screened, and then reshuffled until the desired properties are optimized. Alternatively, fragments of a given gene may be recombined with fragments of homologous genes in the same gene family, either from the same or different species, thereby maximizing the genetic diversity of multiple naturally occurring genes in a directed and controllable manner.

A "variant" of a particular polypeptide sequence is defined as a polypeptide sequence having at least 40% sequence identity to the particular polypeptide sequence over a certain length of one of the polypeptide sequences using blastp with the "BLAST 2 Sequences" tool Version 2.0.9 (May-07-1999) set at default parameters. Such a pair of polypeptides may show, for example, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% or greater sequence identity over a certain defined length of one of the polypeptides.

## THE INVENTION

In a particular embodiment, cDNA sequences derived from human tissues and cell lines were aligned based on nucleotide sequence identity and assembled into "consensus" or "template" sequences which are designated by the template identification numbers (template IDs) in column 2 of Table 1. The sequence identification numbers (SEQ ID NO:s) corresponding to the template IDs are shown in column 1. The template sequences have similarity to GenBank sequences, or "hits," as designated by the GI Numbers in column 3. The statistical probability of each GenBank hit is indicated by a probability score in column 4, and the functional annotation corresponding to each GenBank hit is listed in column 5.

The invention incorporates the nucleic acid sequences of these templates as disclosed in the Sequence Listing and the use of these sequences in the diagnosis and treatment of disease states characterized by defects in human molecules. The invention further utilizes these sequences in hybridization and amplification technologies, and in particular, in technologies which assess gene expression patterns correlated with specific cells or tissues and their responses *in vivo* or *in vitro* to pharmaceutical agents, toxins, and other treatments. In this manner, the sequences of the present

invention are used to develop a transcript image for a particular cell or tissue.

### Derivation of Nucleic Acid Sequences

cDNA was isolated from libraries constructed using RNA derived from normal and diseased human tissues and cell lines. The human tissues and cell lines used for cDNA library construction were selected from a broad range of sources to provide a diverse population of cDNAs representative of gene transcription throughout the human body. Descriptions of the human tissues and cell lines used for cDNA library construction are provided in the LIFESEQ database (Incyte Genomics, Inc. (Incyte), Palo Alto CA). Human tissues were broadly selected from, for example, cardiovascular, dermatologic, endocrine, gastrointestinal, hematopoietic/immune system, musculoskeletal, neural, reproductive, and urologic sources.

Cell lines used for cDNA library construction were derived from, for example, leukemic cells, teratocarcinomas, neuroepitheliomas, cervical carcinoma, lung fibroblasts, and endothelial cells. Such cell lines include, for example, THP-1, Jurkat, HUVEC, hNT2, WI38, HeLa, and other cell lines commonly used and available from public depositories (American Type Culture Collection, Manassas VA). Prior to mRNA isolation, cell lines were untreated, treated with a pharmaceutical agent such as 5'-aza-2'-deoxycytidine, treated with an activating agent such as lipopolysaccharide in the case of leukocytic cell lines, or, in the case of endothelial cell lines, subjected to shear stress.

### Sequencing of the cDNAs

Methods for DNA sequencing are well known in the art. Conventional enzymatic methods employ the Klenow fragment of DNA polymerase I, SEQUENASE DNA polymerase (U.S. Biochemical Corporation, Cleveland OH), Taq polymerase (PE Biosystems, Foster City CA), thermostable T7 polymerase (Amersham Pharmacia Biotech, Inc. (Amersham Pharmacia Biotech), Piscataway NJ), or combinations of polymerases and proofreading exonucleases such as those found in the ELONGASE amplification system (Life Technologies Inc. (Life Technologies), Gaithersburg MD), to extend the nucleic acid sequence from an oligonucleotide primer annealed to the DNA template of interest. Methods have been developed for the use of both single-stranded and double-stranded templates. Chain termination reaction products may be electrophoresed on urea-polyacrylamide gels and detected either by autoradiography (for radioisotope-labeled nucleotides) or by fluorescence (for fluorophore-labeled nucleotides). Automated methods for mechanized reaction preparation, sequencing, and analysis using fluorescence detection methods have been developed. Machines used to prepare cDNAs for sequencing can include the MICROLAB 2200 liquid transfer system (Hamilton Company (Hamilton), Reno NV), Peltier thermal cycler (PTC200; MJ Research, Inc. (MJ Research), Watertown MA), and ABI CATALYST 800 thermal cycler (PE Biosystems). Sequencing can be carried out using,

for example, the ABI 373 or 377 (PE Biosystems) or MEGABACE 1000 (Molecular Dynamics, Inc. (Molecular Dynamics), Sunnyvale CA) DNA sequencing systems, or other automated and manual sequencing systems well known in the art.

The nucleotide sequences of the Sequence Listing have been prepared by current, state-of-the-art, automated methods and, as such, may contain occasional sequencing errors or unidentified nucleotides. Such unidentified nucleotides are designated by an N. These infrequent unidentified bases do not represent a hindrance to practicing the invention for those skilled in the art. Several methods employing standard recombinant techniques may be used to correct errors and complete the missing sequence information. (See, e.g., those described in Ausubel, F.M. et al. (1997) Short Protocols in Molecular Biology, John Wiley & Sons, New York NY; and Sambrook, J. et al. (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Plainview NY.)

#### Assembly of cDNA Sequences

Human polynucleotide sequences may be assembled using programs or algorithms well known in the art. Sequences to be assembled are related, wholly or in part, and may be derived from a single or many different transcripts. Assembly of the sequences can be performed using such programs as PHRAP (Phils Revised Assembly Program) and the GELVIEW fragment assembly system (GCG), or other methods known in the art.

Alternatively, cDNA sequences are used as "component" sequences that are assembled into "template" or "consensus" sequences as follows. Sequence chromatograms are processed, verified, and quality scores are obtained using PHRED. Raw sequences are edited using an editing pathway known as Block 1 (See, e.g., the LIFESEQ Assembled User Guide, Incyte Genomics, Palo Alto, CA). A series of BLAST comparisons is performed and low-information segments and repetitive elements (e.g., dinucleotide repeats, Alu repeats, etc.) are replaced by "n's", or masked, to prevent spurious matches. Mitochondrial and ribosomal RNA sequences are also removed. The processed sequences are then loaded into a relational database management system (RDMS) which assigns edited sequences to existing templates, if available. When additional sequences are added into the RDMS, a process is initiated which modifies existing templates or creates new templates from works in progress (i.e., nonfinal assembled sequences) containing queued sequences or the sequences themselves. After the new sequences have been assigned to templates, the templates can be merged into bins. If multiple templates exist in one bin, the bin can be split and the templates reannotated.

Once gene bins have been generated based upon sequence alignments, bins are "clone joined" based upon clone information. Clone joining occurs when the 5' sequence of one clone is present in one bin and the 3' sequence from the same clone is present in a different bin, indicating that the two bins should be merged into a single bin. Only bins which share at least two different clones are merged.

A resultant template sequence may contain either a partial or a full length open reading frame, or all or part of a genetic regulatory element. This variation is due in part to the fact that the full length cDNAs of many genes are several hundred, and sometimes several thousand, bases in length. With current technology, cDNAs comprising the coding regions of large genes cannot be cloned because of vector limitations, incomplete reverse transcription of the mRNA, or incomplete "second strand" synthesis. Template sequences may be extended to include additional contiguous sequences derived from the parent RNA transcript using a variety of methods known to those of skill in the art. Extension may thus be used to achieve the full length coding sequence of a gene.

#### 10 Analysis of the cDNA Sequences

The cDNA sequences are analyzed using a variety of programs and algorithms which are well known in the art. (See, e.g., Ausubel, 1997, supra, Chapter 7.7; Meyers, R.A. (Ed.) (1995) Molecular Biology and Biotechnology, Wiley VCH, New York NY, pp. 856-853; and Table 5.) These analyses comprise both reading frame determinations, e.g., based on triplet codon periodicity for particular organisms (Fickett, J.W. (1982) *Nucleic Acids Res.* 10:5303-5318); analyses of potential start and stop codons; and homology searches.

Computer programs known to those of skill in the art for performing computer-assisted searches for amino acid and nucleic acid sequence similarity, include, for example, Basic Local Alignment Search Tool (BLAST; Altschul, S.F. (1993) *J. Mol. Evol.* 36:290-300; Altschul, S.F. et al. (1990) *J. Mol. Biol.* 215:403-410). BLAST is especially useful in determining exact matches and comparing two sequence fragments of arbitrary but equal lengths, whose alignment is locally maximal and for which the alignment score meets or exceeds a threshold or cutoff score set by the user (Karlin, S. et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:841-845). Using an appropriate search tool (e.g., BLAST or HMM), GenBank, SwissProt, BLOCKS, PFAM and other databases may be searched for sequences containing regions of homology to a query dithp or DITHP of the present invention.

Other approaches to the identification, assembly, storage, and display of nucleotide and polypeptide sequences are provided in "Relational Database for Storing Biomolecule Information," U.S.S.N. 08/947,845, filed October 9, 1997; "Project-Based Full-Length Biomolecular Sequence Database," U.S.S.N. 08/811,758, filed March 6, 1997; and "Relational Database and System for Storing Information Relating to Biomolecular Sequences," U.S.S.N. 09/034,807, filed March 4, 1998, all of which are incorporated by reference herein in their entirety.

Protein hierarchies can be assigned to the putative encoded polypeptide based on, e.g., motif, BLAST, or biological analysis. Methods for assigning these hierarchies are described, for example, in "Database System Employing Protein Function Hierarchies for Viewing Biomolecular Sequence Data," U.S.S.N. 08/812,290, filed March 6, 1997, incorporated herein by reference.

### Identification of Human Diagnostic and Therapeutic Molecules Encoded by dithp

The identities of the DITHP encoded by the dithp of the present invention were obtained by analysis of the assembled cDNA sequences. SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5 encode, for example, human enzyme molecules. SEQ ID NO:6 and SEQ ID NO:7 encode, for example, receptor molecules. SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12 encode, for example, intracellular signaling molecules. SEQ ID NO:13 encodes, for example, a membrane transport molecule. SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, and SEQ ID NO:20 encode, for example, nucleic acid synthesis and modification molecules. SEQ ID NO:21 and SEQ ID NO:22 encode, for example, adhesion molecules. SEQ ID NO:23 and SEQ ID NO:24 encode, for example, electron transfer associated molecules. SEQ ID NO:25 encodes, for example, a secreted/extracellular matrix molecule. SEQ ID NO:26 and SEQ ID NO:27 encode, for example, cytoskeletal molecules. SEQ ID NO:28 and SEQ ID NO:29 encode, for example, cell membrane molecules. SEQ ID NO:30 and SEQ ID NO:31 encode, for example, ribosomal molecules. SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, and SEQ ID NO:43 encode, for example, transcription factor molecules. SEQ ID NO:44, SEQ ID NO:45, and SEQ ID NO:46 encode, for example, organelle associated molecules. SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, and SEQ ID NO:50 encode, for example, biochemical pathway molecules. SEQ ID NO:51 and SEQ ID NO:52 encode, for example, molecules associated with growth and development.

### Sequences of Human Diagnostic and Therapeutic Molecules

The dithp of the present invention may be used for a variety of diagnostic and therapeutic purposes. For example, a dithp may be used to diagnose a particular condition, disease, or disorder associated with human molecules. Such conditions, diseases, and disorders include, but are not limited to, a cell proliferative disorder, such as actinic keratosis, arteriosclerosis, atherosclerosis, bursitis, cirrhosis, hepatitis, mixed connective tissue disease (MCTD), myelofibrosis, paroxysmal nocturnal hemoglobinuria, polycythemia vera, psoriasis, primary thrombocythemia, and cancers including adenocarcinoma, leukemia, lymphoma, melanoma, myeloma, sarcoma, teratocarcinoma, and, in particular, a cancer of the adrenal gland, bladder, bone, bone marrow, brain, breast, cervix, gall bladder, ganglia, gastrointestinal tract, heart, kidney, liver, lung, muscle, ovary, pancreas, parathyroid, penis, prostate, salivary glands, skin, spleen, testis, thymus, thyroid, and uterus; an autoimmune/inflammatory disorder, such as inflammation, actinic keratosis, acquired immunodeficiency syndrome (AIDS), Addison's disease, adult respiratory distress syndrome,

allergies, ankylosing spondylitis, amyloidosis, anemia, arteriosclerosis, asthma, atherosclerosis, autoimmune hemolytic anemia, autoimmune thyroiditis, bronchitis, bursitis, cholecystitis, cirrhosis, contact dermatitis, Crohn's disease, atopic dermatitis, dermatomyositis, diabetes mellitus, emphysema, erythroblastosis fetalis, erythema nodosum, atrophic gastritis, glomerulonephritis,

5 Goodpasture's syndrome, gout, Graves' disease, Hashimoto's thyroiditis, paroxysmal nocturnal hemoglobinuria, hepatitis, hyper eosinophilia, irritable bowel syndrome, episodic lymphopenia with lymphocytotoxins, mixed connective tissue disease (MCTD), multiple sclerosis, myasthenia gravis, myocardial or pericardial inflammation, myofibrosis, osteoarthritis, osteoporosis, pancreatitis, polycythemia vera, polymyositis, psoriasis, Reiter's syndrome, rheumatoid arthritis, scleroderma,

10 Sjögren's syndrome, systemic anaphylaxis, systemic lupus erythematosus, systemic sclerosis, primary thrombocythemia, thrombocytopenic purpura, ulcerative colitis, uveitis, Werner syndrome, complications of cancer, hemodialysis, and extracorporeal circulation, trauma, and hematopoietic cancer including lymphoma, leukemia, and myeloma; an infection caused by a viral agent classified as adenovirus, arenavirus, bunyavirus, calicivirus, coronavirus, filovirus, hepadnavirus, herpesvirus,

15 flavivirus, orthomyxovirus, parvovirus, papovavirus, paramyxovirus, picornavirus, poxvirus, reovirus, rotavirus, rhabdovirus, or togavirus; an infection caused by a bacterial agent classified as pneumococcus, staphylococcus, streptococcus, bacillus, corynebacterium, clostridium, meningococcus, gonococcus, listeria, moraxella, kingella, haemophilus, legionella, bordetella, gram-negative enterobacterium including shigella, salmonella, or campylobacter, pseudomonas, vibrio,

20 brucella, francisella, yersinia, bartonella, norcardium, actinomyces, mycobacterium, spirochaetale, rickettsia, chlamydia, or mycoplasma; an infection caused by a fungal agent classified as aspergillus, blastomyces, dermatophytes, cryptococcus, coccidioides, malassezia, histoplasma, or other mycosis-causing fungal agent; and an infection caused by a parasite classified as plasmodium or malaria-causing, parasitic entamoeba, leishmania, trypanosoma, toxoplasma, pneumocystis carinii, intestinal

25 protozoa such as giardia, trichomonas, tissue nematode such as trichinella, intestinal nematode such as ascaris, lymphatic filarial nematode, trematode such as schistosoma, and cestode such as tapeworm; a developmental disorder such as renal tubular acidosis, anemia, Cushing's syndrome, achondroplastic dwarfism, Duchenne and Becker muscular dystrophy, epilepsy, gonadal dysgenesis, WAGR syndrome (Wilms' tumor, aniridia, genitourinary abnormalities, and mental retardation),

30 Smith-Magenis syndrome, myelodysplastic syndrome, hereditary mucocutaneous dysplasia, hereditary keratodermas, hereditary neuropathies such as Charcot-Marie-Tooth disease and neurofibromatosis, hypothyroidism, hydrocephalus, seizure disorders such as Sydenham's chorea and cerebral palsy, spina bifida, anencephaly, craniorachischisis, congenital glaucoma, cataract, and sensorineural hearing loss; an endocrine disorder such as a disorder of the hypothalamus and/or pituitary resulting from

35 lesions such as a primary brain tumor, adenoma, infarction associated with pregnancy,

- hypophysectomy, aneurysm, vascular malformation, thrombosis, infection, immunological disorder, and complication due to head trauma; a disorder associated with hypopituitarism including hypogonadism, Sheehan syndrome, diabetes insipidus, Kallman's disease, Hand-Schuller-Christian disease, Letterer-Siwe disease, sarcoidosis, empty sella syndrome, and dwarfism; a disorder associated with
- 5 hyperpituitarism including acromegaly, gigantism, and syndrome of inappropriate antidiuretic hormone (ADH) secretion (SIADH) often caused by benign adenoma; a disorder associated with hypothyroidism including goiter, myxedema, acute thyroiditis associated with bacterial infection, subacute thyroiditis associated with viral infection, autoimmune thyroiditis (Hashimoto's disease), and cretinism; a disorder associated with hyperthyroidism including thyrotoxicosis and its various forms, Grave's disease,
- 10 pretibial myxedema, toxic multinodular goiter, thyroid carcinoma, and Plummer's disease; a disorder associated with hyperparathyroidism including Conn disease (chronic hypercalcemia); a pancreatic disorder such as Type I or Type II diabetes mellitus and associated complications; a disorder associated with the adrenals such as hyperplasia, carcinoma, or adenoma of the adrenal cortex, hypertension associated with alkalosis, amyloidosis, hypokalemia, Cushing's disease, Liddle's syndrome, and
- 15 Arnold-Healy-Gordon syndrome, pheochromocytoma tumors, and Addison's disease; a disorder associated with gonadal steroid hormones such as: in women, abnormal prolactin production, infertility, endometriosis, perturbation of the menstrual cycle, polycystic ovarian disease, hyperprolactinemia, isolated gonadotropin deficiency, amenorrhea, galactorrhea, hermaphroditism, hirsutism and virilization, breast cancer, and, in post-menopausal women, osteoporosis; and, in men,
- 20 Leydig cell deficiency, male climacteric phase, and germinal cell aplasia, a hypergonadal disorder associated with Leydig cell tumors, androgen resistance associated with absence of androgen receptors, syndrome of 5  $\alpha$ -reductase, and gynecomastia; a metabolic disorder such as Addison's disease, cerebrotendinous xanthomatosis, congenital adrenal hyperplasia, coumarin resistance, cystic fibrosis, diabetes, fatty hepatocirrhosis, fructose-1,6-diphosphatase deficiency, galactosemia, goiter,
- 25 glucagonoma, glycogen storage diseases, hereditary fructose intolerance, hyperadrenalism, hypoadrenalism, hyperparathyroidism, hypoparathyroidism, hypercholesterolemia, hyperthyroidism, hypoglycemia, hypothyroidism, hyperlipidemia, hyperlipemia, lipid myopathies, lipodystrophies, lysosomal storage diseases, mannosidosis, neuraminidase deficiency, obesity, pentosuria phenylketonuria, pseudovitamin D-deficiency rickets; disorders of carbohydrate metabolism such as
- 30 congenital type II dyserythropoietic anemia, diabetes, insulin-dependent diabetes mellitus, non-insulin-dependent diabetes mellitus, fructose-1,6-diphosphatase deficiency, galactosemia, glucagonoma, hereditary fructose intolerance, hypoglycemia, mannosidosis, neuraminidase deficiency, obesity, galactose epimerase deficiency, glycogen storage diseases, lysosomal storage diseases, fructosuria, pentosuria, and inherited abnormalities of pyruvate metabolism; disorders of
- 35 lipid metabolism such as fatty liver, cholestasis, primary biliary cirrhosis, carnitine deficiency,

- carnitine palmitoyltransferase deficiency, myoadenylate deaminase deficiency, hypertriglyceridemia, lipid storage disorders such as Fabry's disease, Gaucher's disease, Niemann-Pick's disease, metachromatic leukodystrophy, adrenoleukodystrophy, GM<sub>2</sub> gangliosidosis, and ceroid lipofuscinosis, abetalipoproteinemia, Tangier disease, hyperlipoproteinemia, diabetes mellitus,
- 5 lipodystrophy, lipomatosis, acute panniculitis, disseminated fat necrosis, adiposis dolorosa, lipoid adrenal hyperplasia, minimal change disease, lipomas, atherosclerosis, hypercholesterolemia, hypercholesterolemia with hypertriglyceridemia, primary hypoparathyroidism, hypothyroidism, renal disease, liver disease, lecithin:cholesterol acyltransferase deficiency, cerebrotendinous xanthomatosis, sitosterolemia, hypocholesterolemia, Tay-Sachs disease, Sandhoff's disease,
- 10 hyperlipidemia, hyperlipemia, lipid myopathies, and obesity; and disorders of copper metabolism such as Menke's disease, Wilson's disease, and Ehlers-Danlos syndrome type IX; a neurological disorder such as epilepsy, ischemic cerebrovascular disease, stroke, cerebral neoplasms, Alzheimer's disease, Pick's disease, Huntington's disease, dementia, Parkinson's disease and other extrapyramidal disorders, amyotrophic lateral sclerosis and other motor neuron disorders, progressive neural muscular
- 15 atrophy, retinitis pigmentosa, hereditary ataxias, multiple sclerosis and other demyelinating diseases, bacterial and viral meningitis, brain abscess, subdural empyema, epidural abscess, suppurative intracranial thrombophlebitis, myelitis and radiculitis, viral central nervous system disease, prion diseases including kuru, Creutzfeldt-Jakob disease, and Gerstmann-Strausler-Scheinker syndrome, fatal familial insomnia, nutritional and metabolic diseases of the nervous system, neurofibromatosis,
- 20 tuberous sclerosis, cerebelloretinal hemangioblastomatosis, encephalotrigeminal syndrome, mental retardation and other developmental disorder of the central nervous system, cerebral palsy, a neuroskeletal disorder, an autonomic nervous system disorder, a cranial nerve disorder, a spinal cord disease, muscular dystrophy and other neuromuscular disorder, a peripheral nervous system disorder, dermatomyositis and polymyositis, inherited, metabolic, endocrine, and toxic myopathy, myasthenia
- 25 gravis, periodic paralysis, a mental disorder including mood, anxiety, and schizophrenic disorders, seasonal affective disorder (SAD), akathisia, amnesia, catatonia, diabetic neuropathy, tardive dyskinesia, dystonias, paranoid psychoses, postherpetic neuralgia, and Tourette's disorder; a gastrointestinal disorder including ulcerative colitis, gastric and duodenal ulcers, cystinuria, dibasicaminoaciduria, hypercystinuria, lysinuria, hartnup disease, tryptophan malabsorption,
- 30 methionine malabsorption, histidinuria, iminoglycinuria, dicarboxylicaminoaciduria, cystinosis, renal glycosuria, hypouricemia, familial hypophosphatemic rickets, congenital chloridorrhea, distal renal tubular acidosis, Menkes' disease, Wilson's disease, lethal diarrhea, juvenile pernicious anemia, folate malabsorption, adrenoleukodystrophy, hereditary myoglobinuria, and Zellweger syndrome; a
- 35 Becker's muscular dystrophy, Bell's palsy, Charcot-Marie Tooth disease, diabetes mellitus, diabetes



insipidus, diabetic neuropathy, Duchenne muscular dystrophy, hyperkalemic periodic paralysis, normokalemic periodic paralysis, Parkinson's disease, malignant hyperthermia, multidrug resistance, myasthenia gravis, myotonic dystrophy, catatonia, tardive dyskinesia, dystonias, peripheral neuropathy, cerebral neoplasms, prostate cancer, cardiac disorders associated with transport, e.g.,

5 angina, bradyarrhythmia, tachyarrhythmia, hypertension, Long QT syndrome, myocarditis, cardiomyopathy, nemaline myopathy, centronuclear myopathy, lipid myopathy, mitochondrial myopathy, thyrotoxic myopathy, ethanol myopathy, dermatomyositis, inclusion body myositis, infectious myositis, and polymyositis, neurological disorders associated with transport, e.g., Alzheimer's disease, amnesia, bipolar disorder, dementia, depression, epilepsy, Tourette's disorder,

10 paranoid psychoses, and schizophrenia, and other disorders associated with transport, e.g., neurofibromatosis, postherpetic neuralgia, trigeminal neuropathy, sarcoidosis, sickle cell anemia, cataracts, infertility, pulmonary artery stenosis, sensorineural autosomal deafness, hyperglycemia, hypoglycemia, Grave's disease, goiter, glucose-galactose malabsorption syndrome, hypercholesterolemia, Cushing's disease, and Addison's disease; and a connective tissue disorder

15 such as osteogenesis imperfecta, Ehlers-Danlos syndrome, chondrodysplasias, Marfan syndrome, Alport syndrome, familial aortic aneurysm, achondroplasia, mucopolysaccharidoses, osteoporosis, osteopetrosis, Paget's disease, rickets, osteomalacia, hyperparathyroidism, renal osteodystrophy, osteonecrosis, osteomyelitis, osteoma, osteoid osteoma, osteoblastoma, osteosarcoma, osteochondroma, chondroma, chondroblastoma, chondromyxoid fibroma, chondrosarcoma, fibrous

20 cortical defect, nonossifying fibroma, fibrous dysplasia, fibrosarcoma, malignant fibrous histiocytoma, Ewing's sarcoma, primitive neuroectodermal tumor, giant cell tumor, osteoarthritis, rheumatoid arthritis, ankylosing spondyloarthritis, Reiter's syndrome, psoriatic arthritis, enteropathic arthritis, infectious arthritis, gout, gouty arthritis, calcium pyrophosphate crystal deposition disease, ganglion, synovial cyst, villonodular synovitis, systemic sclerosis, Dupuytren's contracture, hepatic

25 fibrosis, lupus erythematosus, mixed connective tissue disease, epidermolysis bullosa simplex, bullous congenital ichthyosiform erythroderma (epidermolytic hyperkeratosis), non-epidermolytic and epidermolytic palmoplantar keratoderma, ichthyosis bullosa of Siemens, pachyonychia congenita, and white sponge nevus. The dithp can be used to detect the presence of, or to quantify the amount of, a dithp-related polynucleotide in a sample. This information is then compared to information obtained

30 from appropriate reference samples, and a diagnosis is established. Alternatively, a polynucleotide complementary to a given dithp can inhibit or inactivate a therapeutically relevant gene related to the dithp.

#### Analysis of dithp Expression Patterns

35 The expression of dithp may be routinely assessed by hybridization-based methods to

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determine, for example, the tissue-specificity, disease-specificity, or developmental stage-specificity of dithp expression. For example, the level of expression of dithp may be compared among different cell types or tissues, among diseased and normal cell types or tissues, among cell types or tissues at different developmental stages, or among cell types or tissues undergoing various treatments. This type of analysis is useful, for example, to assess the relative levels of dithp expression in fully or partially differentiated cells or tissues, to determine if changes in dithp expression levels are correlated with the development or progression of specific disease states, and to assess the response of a cell or tissue to a specific therapy, for example, in pharmacological or toxicological studies. Methods for the analysis of dithp expression are based on hybridization and amplification technologies and include membrane-based procedures such as northern blot analysis, high-throughput procedures that utilize, for example, microarrays, and PCR-based procedures.

#### Hybridization and Genetic Analysis

The dithp, their fragments, or complementary sequences, may be used to identify the presence of and/or to determine the degree of similarity between two (or more) nucleic acid sequences. The dithp may be hybridized to naturally occurring or recombinant nucleic acid sequences under appropriately selected temperatures and salt concentrations. Hybridization with a probe based on the nucleic acid sequence of at least one of the dithp allows for the detection of nucleic acid sequences, including genomic sequences, which are identical or related to the dithp of the Sequence Listing. Probes may be selected from non-conserved or unique regions of at least one of the polynucleotides of SEQ ID NO:1-52 and tested for their ability to identify or amplify the target nucleic acid sequence using standard protocols.

Polynucleotide sequences that are capable of hybridizing, in particular, to those shown in SEQ ID NO:1-52 and fragments thereof, can be identified using various conditions of stringency. (See, e.g., Wahl, G.M. and S.L. Berger (1987) *Methods Enzymol.* 152:399-407; Kimmel, A.R. (1987) *Methods Enzymol.* 152:507-511.) Hybridization conditions are discussed in "Definitions."

A probe for use in Southern or northern hybridization may be derived from a fragment of a dithp sequence, or its complement, that is up to several hundred nucleotides in length and is either single-stranded or double-stranded. Such probes may be hybridized in solution to biological materials such as plasmids, bacterial, yeast, or human artificial chromosomes, cleared or sectioned tissues, or to artificial substrates containing dithp. Microarrays are particularly suitable for identifying the presence of and detecting the level of expression for multiple genes of interest by examining gene expression correlated with, e.g., various stages of development, treatment with a drug or compound, or disease progression. An array analogous to a dot or slot blot may be used to arrange and link polynucleotides to the surface of a substrate using one or more of the following: mechanical (vacuum), chemical,

thermal, or UV bonding procedures. Such an array may contain any number of dithp and may be produced by hand or by using available devices, materials, and machines.

Microarrays may be prepared, used, and analyzed using methods known in the art. (See, e.g., Brennan, T.M. et al. (1995) U.S. Patent No. 5,474,796; Schena, M. et al. (1996) Proc. Natl. Acad. Sci. USA 93:10614-10619; Baldeschweiler et al. (1995) PCT application WO95/251116; Shalon, D. et al. (1995) PCT application WO95/35505; Heller, R.A. et al. (1997) Proc. Natl. Acad. Sci. USA 94:2150-2155; and Heller, M.J. et al. (1997) U.S. Patent No. 5,605,662.)

Probes may be labeled by either PCR or enzymatic techniques using a variety of commercially available reporter molecules. For example, commercial kits are available for radioactive and chemiluminescent labeling (Amersham Pharmacia Biotech) and for alkaline phosphatase labeling (Life Technologies). Alternatively, dithp may be cloned into commercially available vectors for the production of RNA probes. Such probes may be transcribed in the presence of at least one labeled nucleotide (e.g., <sup>32</sup>P-ATP, Amersham Pharmacia Biotech).

Additionally the polynucleotides of SEQ ID NO:1-52 or suitable fragments thereof can be used to isolate full length cDNA sequences utilizing hybridization and/or amplification procedures well known in the art, e.g., cDNA library screening, PCR amplification, etc. The molecular cloning of such full length cDNA sequences may employ the method of cDNA library screening with probes using the hybridization, stringency, washing, and probing strategies described above and in Ausubel, supra, Chapters 3, 5, and 6. These procedures may also be employed with genomic libraries to isolate genomic sequences of dithp in order to analyze, e.g., regulatory elements.

### Genetic Mapping

Gene identification and mapping are important in the investigation and treatment of almost all conditions, diseases, and disorders. Cancer, cardiovascular disease, Alzheimer's disease, arthritis, diabetes, and mental illnesses are of particular interest. Each of these conditions is more complex than the single gene defects of sickle cell anemia or cystic fibrosis, with select groups of genes being predictive of predisposition for a particular condition, disease, or disorder. For example, cardiovascular disease may result from malfunctioning receptor molecules that fail to clear cholesterol from the bloodstream, and diabetes may result when a particular individual's immune system is activated by an infection and attacks the insulin-producing cells of the pancreas. In some studies, Alzheimer's disease has been linked to a gene on chromosome 21; other studies predict a different gene and location. Mapping of disease genes is a complex and reiterative process and generally proceeds from genetic linkage analysis to physical mapping.

As a condition is noted among members of a family, a genetic linkage map traces parts of chromosomes that are inherited in the same pattern as the condition. Statistics link the inheritance of

particular conditions to particular regions of chromosomes, as defined by RFLP or other markers. (See, for example, Lander, E. S. and Botstein, D. (1986) Proc. Natl. Acad. Sci. USA 83:7353-7357.) Occasionally, genetic markers and their locations are known from previous studies. More often, however, the markers are simply stretches of DNA that differ among individuals. Examples of genetic linkage maps can be found in various scientific journals or at the Online Mendelian Inheritance in Man (OMIM) World Wide Web site.

In another embodiment of the invention, dithp sequences may be used to generate hybridization probes useful in chromosomal mapping of naturally occurring genomic sequences. Either coding or noncoding sequences of dithp may be used, and in some instances, noncoding sequences may be preferable over coding sequences. For example, conservation of a dithp coding sequence among members of a multi-gene family may potentially cause undesired cross hybridization during chromosomal mapping. The sequences may be mapped to a particular chromosome, to a specific region of a chromosome, or to artificial chromosome constructions, e.g., human artificial chromosomes (HACs), yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), bacterial P1 constructions, or single chromosome cDNA libraries. (See, e.g., Harrington, J.J. et al. (1997) Nat. Genet. 15:345-355; Price, C.M. (1993) Blood Rev. 7:127-134; and Trask, B.J. (1991) Trends Genet. 7:149-154.)

Fluorescent in situ hybridization (FISH) may be correlated with other physical chromosome mapping techniques and genetic map data. (See, e.g., Meyers, supra, pp. 965-968.) Correlation between the location of dithp on a physical chromosomal map and a specific disorder, or a predisposition to a specific disorder, may help define the region of DNA associated with that disorder. The dithp sequences may also be used to detect polymorphisms that are genetically linked to the inheritance of a particular condition, disease, or disorder.

In situ hybridization of chromosomal preparations and genetic mapping techniques, such as linkage analysis using established chromosomal markers, may be used for extending existing genetic maps. Often the placement of a gene on the chromosome of another mammalian species, such as mouse, may reveal associated markers even if the number or arm of the corresponding human chromosome is not known. These new marker sequences can be mapped to human chromosomes and may provide valuable information to investigators searching for disease genes using positional cloning or other gene discovery techniques. Once a disease or syndrome has been crudely correlated by genetic linkage with a particular genomic region, e.g., ataxia-telangiectasia to 11q22-23, any sequences mapping to that area may represent associated or regulatory genes for further investigation. (See, e.g., Gatti, R.A. et al. (1988) Nature 336:577-580.) The nucleotide sequences of the subject invention may also be used to detect differences in chromosomal architecture due to translocation, inversion, etc., among normal, carrier, or affected individuals.

Once a disease-associated gene is mapped to a chromosomal region, the gene must be cloned in order to identify mutations or other alterations (e.g., translocations or inversions) that may be correlated with disease. This process requires a physical map of the chromosomal region containing the disease-gene of interest along with associated markers. A physical map is necessary for determining the

5 nucleotide sequence of and order of marker genes on a particular chromosomal region. Physical mapping techniques are well known in the art and require the generation of overlapping sets of cloned DNA fragments from a particular organelle, chromosome, or genome. These clones are analyzed to reconstruct and catalog their order. Once the position of a marker is determined, the DNA from that region is obtained by consulting the catalog and selecting clones from that region. The gene of interest

10 is located through positional cloning techniques using hybridization or similar methods.

#### Diagnostic Uses

The dithp of the present invention may be used to design probes useful in diagnostic assays. Such assays, well known to those skilled in the art, may be used to detect or confirm conditions,

15 disorders, or diseases associated with abnormal levels of dithp expression. Labeled probes developed from dithp sequences are added to a sample under hybridizing conditions of desired stringency. In some instances, dithp, or fragments or oligonucleotides derived from dithp, may be used as primers in amplification steps prior to hybridization. The amount of hybridization complex formed is quantified and compared with standards for that cell or tissue. If dithp expression varies significantly from the

20 standard, the assay indicates the presence of the condition, disorder, or disease. Qualitative or quantitative diagnostic methods may include northern, dot blot, or other membrane or dip-stick based technologies or multiple-sample format technologies such as PCR, enzyme-linked immunosorbent assay (ELISA)-like, pin, or chip-based assays.

The probes described above may also be used to monitor the progress of conditions, disorders,

25 or diseases associated with abnormal levels of dithp expression, or to evaluate the efficacy of a particular therapeutic treatment. The candidate probe may be identified from the dithp that are specific to a given human tissue and have not been observed in GenBank or other genome databases. Such a probe may be used in animal studies, preclinical tests, clinical trials, or in monitoring the treatment of an individual patient. In a typical process, standard expression is established by methods well known in

30 the art for use as a basis of comparison, samples from patients affected by the disorder or disease are combined with the probe to evaluate any deviation from the standard profile, and a therapeutic agent is administered and effects are monitored to generate a treatment profile. Efficacy is evaluated by determining whether the expression progresses toward or returns to the standard normal pattern. Treatment profiles may be generated over a period of several days or several months. Statistical

35 methods well known to those skilled in the art may be used to determine the significance of such

therapeutic agents.

The polynucleotides are also useful for identifying individuals from minute biological samples, for example, by matching the RFLP pattern of a sample's DNA to that of an individual's DNA. The polynucleotides of the present invention can also be used to determine the actual base-by-base DNA sequence of selected portions of an individual's genome. These sequences can be used to prepare PCR primers for amplifying and isolating such selected DNA, which can then be sequenced. Using this technique, an individual can be identified through a unique set of DNA sequences. Once a unique ID database is established for an individual, positive identification of that individual can be made from extremely small tissue samples.

In a particular aspect, oligonucleotide primers derived from the dithp of the invention may be used to detect single nucleotide polymorphisms (SNPs). SNPs are substitutions, insertions and deletions that are a frequent cause of inherited or acquired genetic disease in humans. Methods of SNP detection include, but are not limited to, single-stranded conformation polymorphism (SSCP) and fluorescent SSCP (fSSCP) methods. In SSCP, oligonucleotide primers derived from the polynucleotide sequences encoding DITHP are used to amplify DNA using the polymerase chain reaction (PCR). The DNA may be derived, for example, from diseased or normal tissue, biopsy samples, bodily fluids, and the like. SNPs in the DNA cause differences in the secondary and tertiary structures of PCR products in single-stranded form, and these differences are detectable using gel electrophoresis in non-denaturing gels. In fSSCP, the oligonucleotide primers are fluorescently labeled, which allows detection of the amplimers in high-throughput equipment such as DNA sequencing machines. Additionally, sequence database analysis methods, termed *in silico* SNP (isSNP), are capable of identifying polymorphisms by comparing the sequences of individual overlapping DNA fragments which assemble into a common consensus sequence. These computer-based methods filter out sequence variations due to laboratory preparation of DNA and sequencing errors using statistical models and automated analyses of DNA sequence chromatograms. In the alternative, SNPs may be detected and characterized by mass spectrometry using, for example, the high throughput MASSARRAY system (Sequenom, Inc., San Diego CA).

DNA-based identification techniques are critical in forensic technology. DNA sequences taken from very small biological samples such as tissues, e.g., hair or skin, or body fluids, e.g., blood, saliva, semen, etc., can be amplified using, e.g., PCR, to identify individuals. (See, e.g., Erlich, H. (1992) PCR Technology, Freeman and Co., New York, NY). Similarly, polynucleotides of the present invention can be used as polymorphic markers.

There is also a need for reagents capable of identifying the source of a particular tissue. Appropriate reagents can comprise, for example, DNA probes or primers prepared from the sequences of the present invention that are specific for particular tissues. Panels of such reagents can identify

tissue by species and/or by organ type. In a similar fashion, these reagents can be used to screen tissue cultures for contamination.

The polynucleotides of the present invention can also be used as molecular weight markers on nucleic acid gels or Southern blots, as diagnostic probes for the presence of a specific mRNA in a particular cell type, in the creation of subtracted cDNA libraries which aid in the discovery of novel polynucleotides, in selection and synthesis of oligomers for attachment to an array or other support, and as an antigen to elicit an immune response.

#### Disease Model Systems Using dithp

The dithp of the invention or their mammalian homologs may be “knocked out” in an animal model system using homologous recombination in embryonic stem (ES) cells. Such techniques are well known in the art and are useful for the generation of animal models of human disease. (See, e.g., U.S. Patent Number 5,175,383 and U.S. Patent Number 5,767,337.) For example, mouse ES cells, such as the mouse 129/SvJ cell line, are derived from the early mouse embryo and grown in culture. The ES cells are transformed with a vector containing the gene of interest disrupted by a marker gene, e.g., the neomycin phosphotransferase gene (neo; Capecchi, M.R. (1989) Science 244:1288-1292). The vector integrates into the corresponding region of the host genome by homologous recombination.

Alternatively, homologous recombination takes place using the Cre-loxP system to knockout a gene of interest in a tissue- or developmental stage-specific manner (Marth, J.D. (1996) Clin. Invest. 97:1999-2002; Wagner, K.U. et al. (1997) Nucleic Acids Res. 25:4323-4330). Transformed ES cells are identified and microinjected into mouse cell blastocysts such as those from the C57BL/6 mouse strain. The blastocysts are surgically transferred to pseudopregnant dams, and the resulting chimeric progeny are genotyped and bred to produce heterozygous or homozygous strains. Transgenic animals thus generated may be tested with potential therapeutic or toxic agents.

The dithp of the invention may also be manipulated in vitro in ES cells derived from human blastocysts. Human ES cells have the potential to differentiate into at least eight separate cell lineages including endoderm, mesoderm, and ectodermal cell types. These cell lineages differentiate into, for example, neural cells, hematopoietic lineages, and cardiomyocytes (Thomson, J.A. et al. (1998) Science 282:1145-1147).

The dithp of the invention can also be used to create “knockin” humanized animals (pigs) or transgenic animals (mice or rats) to model human disease. With knockin technology, a region of dithp is injected into animal ES cells, and the injected sequence integrates into the animal cell genome. Transformed cells are injected into blastulae, and the blastulae are implanted as described above. Transgenic progeny or inbred lines are studied and treated with potential pharmaceutical agents to obtain information on treatment of a human disease. Alternatively, a mammal inbred to overexpress

dithp, resulting, e.g., in the secretion of DITHP in its milk, may also serve as a convenient source of that protein (Janne, J. et al. (1998) *Biotechnol. Annu. Rev.* 4:55-74).

### Screening Assays

DITHP encoded by polynucleotides of the present invention may be used to screen for molecules that bind to or are bound by the encoded polypeptides. The binding of the polypeptide and the molecule may activate (agonist), increase, inhibit (antagonist), or decrease activity of the polypeptide or the bound molecule. Examples of such molecules include antibodies, oligonucleotides, proteins (e.g., receptors), or small molecules.

Preferably, the molecule is closely related to the natural ligand of the polypeptide, e.g., a ligand or fragment thereof, a natural substrate, or a structural or functional mimetic. (See, Coligan et al., (1991) Current Protocols in Immunology 1(2): Chapter 5.) Similarly, the molecule can be closely related to the natural receptor to which the polypeptide binds, or to at least a fragment of the receptor, e.g., the active site. In either case, the molecule can be rationally designed using known techniques.

Preferably, the screening for these molecules involves producing appropriate cells which express the polypeptide, either as a secreted protein or on the cell membrane. Preferred cells include cells from mammals, yeast, *Drosophila*, or *E. coli*. Cells expressing the polypeptide or cell membrane fractions which contain the expressed polypeptide are then contacted with a test compound and binding, stimulation, or inhibition of activity of either the polypeptide or the molecule is analyzed.

An assay may simply test binding of a candidate compound to the polypeptide, wherein binding is detected by a fluorophore, radioisotope, enzyme conjugate, or other detectable label. Alternatively, the assay may assess binding in the presence of a labeled competitor.

Additionally, the assay can be carried out using cell-free preparations, polypeptide/molecule affixed to a solid support, chemical libraries, or natural product mixtures. The assay may also simply comprise the steps of mixing a candidate compound with a solution containing a polypeptide, measuring polypeptide/molecule activity or binding, and comparing the polypeptide/molecule activity or binding to a standard.

Preferably, an ELISA assay using, e.g., a monoclonal or polyclonal antibody, can measure polypeptide level in a sample. The antibody can measure polypeptide level by either binding, directly or indirectly, to the polypeptide or by competing with the polypeptide for a substrate.

All of the above assays can be used in a diagnostic or prognostic context. The molecules discovered using these assays can be used to treat disease or to bring about a particular result in a patient (e.g., blood vessel growth) by activating or inhibiting the polypeptide/molecule. Moreover, the assays can discover agents which may inhibit or enhance the production of the polypeptide from suitably manipulated cells or tissues.



### Transcript Imaging

Another embodiment relates to the use of dithp to develop a transcript image of a tissue or cell type. A transcript image is the collective pattern of gene expression by a particular tissue or cell type under given conditions and at a given time. This pattern of gene expression is defined by the number of expressed genes, their abundance, and their function. Thus the dithp of the present invention may be used to develop a transcript image of a tissue or cell type by hybridizing, preferably in a microarray format, the dithp of the present invention to the totality of transcripts or reverse transcripts of a tissue or cell type. The resultant transcript image would provide a profile of gene activity pertaining to human molecules for diagnostics and therapeutics.

Transcript images which profile dithp expression may be generated using transcripts isolated from tissues, cell lines, biopsies, or other biological samples. The transcript image may thus reflect dithp expression in vivo, as in the case of a tissue or biopsy sample, or in vitro, as in the case of a cell line. Transcript images may be used to profile dithp expression in distinct tissue types. This process can be used to determine the activity of human diagnostic and therapeutic molecules in a particular tissue type relative to this activity in a different tissue type. Transcript images may be used to generate a profile of dithp expression characteristic of diseased tissue. Transcript images of tissues before and after treatment may be used for diagnostic purposes, to monitor the progression of disease, and to monitor the efficacy of drug treatments for diseases which affect the activity of human diagnostic and therapeutic molecules.

Transcript images which profile dithp expression may also be used in conjunction with in vitro model systems and preclinical evaluation of pharmaceuticals. Transcript images of cell lines can be used to assess the activity of human diagnostic and therapeutic molecules and/or to identify cell lines that lack or misregulate this activity. Such cell lines may then be treated with pharmaceutical agents, and a transcript image following treatment may indicate the efficacy of these agents in restoring desired levels of this activity. A similar approach may be used to assess the toxicity of pharmaceutical agents as reflected by undesirable changes in the activity of human diagnostic and therapeutic molecules. Candidate pharmaceutical agents may be evaluated by comparing their associated transcript images with those of pharmaceutical agents of known effectiveness.

### Antisense Molecules

The polynucleotides of the present invention are useful in antisense technology. Antisense technology or therapy relies on the modulation of expression of a target protein through the specific binding of an antisense sequence to a target sequence encoding the target protein or directing its expression. (See, e.g., Agrawal, S., ed. (1996) Antisense Therapeutics, Humana Press Inc., Totawa NJ; Alama, A. et al. (1997) *Pharmacol. Res.* 36(3):171-178; Crooke, S.T. (1997) *Adv. Pharmacol.*

40:1-49; Sharma, H.W. and R. Narayanan (1995) Bioessays 17(12):1055-1063; and Lavrosky, Y. et al. (1997) Biochem. Mol. Med. 62(1):11-22.) An antisense sequence is a polynucleotide sequence capable of specifically hybridizing to at least a portion of the target sequence. Antisense sequences bind to cellular mRNA and/or genomic DNA, affecting translation and/or transcription. Antisense sequences can be DNA, RNA, or nucleic acid mimics and analogs. (See, e.g., Rossi, J.J. et al. (1991) Antisense Res. Dev. 1(3):285-288; Lee, R. et al. (1998) Biochemistry 37(3):900-1010; Pardridge, W.M. et al. (1995) Proc. Natl. Acad. Sci. USA 92(12):5592-5596; and Nielsen, P. E. and Haaijma, G. (1997) Chem. Soc. Rev. 96:73-78.) Typically, the binding which results in modulation of expression occurs through hybridization or binding of complementary base pairs. Antisense sequences can also bind to DNA duplexes through specific interactions in the major groove of the double helix.

The polynucleotides of the present invention and fragments thereof can be used as antisense sequences to modify the expression of the polypeptide encoded by dithp. The antisense sequences can be produced ex vivo, such as by using any of the ABI nucleic acid synthesizer series (PE Biosystems) or other automated systems known in the art. Antisense sequences can also be produced biologically, such as by transforming an appropriate host cell with an expression vector containing the sequence of interest. (See, e.g., Agrawal, supra.)

In therapeutic use, any gene delivery system suitable for introduction of the antisense sequences into appropriate target cells can be used. Antisense sequences can be delivered intracellularly in the form of an expression plasmid which, upon transcription, produces a sequence complementary to at least a portion of the cellular sequence encoding the target protein. (See, e.g., Slater, J.E., et al. (1998) J. Allergy Clin. Immunol. 102(3):469-475; and Scanlon, K.J., et al. (1995) 9(13):1288-1296.) Antisense sequences can also be introduced intracellularly through the use of viral vectors, such as retrovirus and adeno-associated virus vectors. (See, e.g., Miller, A.D. (1990) Blood 76:271; Ausubel, F.M. et al. (1995) Current Protocols in Molecular Biology, John Wiley & Sons, New York NY; Uckert, W. and W. Walther (1994) Pharmacol. Ther. 63(3):323-347.) Other gene delivery mechanisms include liposome-derived systems, artificial viral envelopes, and other systems known in the art. (See, e.g., Rossi, J.J. (1995) Br. Med. Bull. 51(1):217-225; Boado, R.J. et al. (1998) J. Pharm. Sci. 87(11):1308-1315; and Morris, M.C. et al. (1997) Nucleic Acids Res. 25(14):2730-2736.)

### 30 Expression

In order to express a biologically active DITHP, the nucleotide sequences encoding DITHP or fragments thereof may be inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for transcriptional and translational control of the inserted coding sequence in a suitable host. Methods which are well known to those skilled in the art may be used to construct expression vectors containing sequences encoding DITHP and appropriate transcriptional and

translational control elements. These methods include in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. (See, e.g., Sambrook, supra, Chapters 4, 8, 16, and 17; and Ausubel, supra, Chapters 9, 10, 13, and 16.)

A variety of expression vector/host systems may be utilized to contain and express sequences encoding DITHP. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with viral expression vectors (e.g., baculovirus); plant cell systems transformed with viral expression vectors (e.g., cauliflower mosaic virus, CaMV, or tobacco mosaic virus, TMV) or with bacterial expression vectors (e.g., Ti or pBR322 plasmids); or animal (mammalian) cell systems. (See, e.g., Sambrook, supra; Ausubel, 1995, supra, Van Heeke, G. and S.M. Schuster (1989) *J. Biol. Chem.* 264:5503-5509; Bitter, G.A. et al. (1987) *Methods Enzymol.* 153:516-544; Scorer, C.A. et al. (1994) *BioTechnology* 12:181-184; Engelhard, E.K. et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:3224-3227; Sandig, V. et al. (1996) *Hum. Gene Ther.* 7:1937-1945; Takamatsu, N. (1987) *EMBO J.* 6:307-311; Coruzzi, G. et al. (1984) *EMBO J.* 3:1671-1680; Broglie, R. et al. (1984) *Science* 224:838-843; Winter, J. et al. (1991) *Results Probl. Cell Differ.* 17:85-105; The McGraw Hill Yearbook of Science and Technology (1992) McGraw Hill, New York NY, pp. 191-196; Logan, J. and T. Shenk (1984) *Proc. Natl. Acad. Sci. USA* 81:3655-3659; and Harrington, J.J. et al. (1997) *Nat. Genet.* 15:345-355.) Expression vectors derived from retroviruses, adenoviruses, or herpes or vaccinia viruses, or from various bacterial plasmids, may be used for delivery of nucleotide sequences to the targeted organ, tissue, or cell population. (See, e.g., Di Nicola, M. et al. (1998) *Cancer Gen. Ther.* 5(6):350-356; Yu, M. et al., (1993) *Proc. Natl. Acad. Sci. USA* 90(13):6340-6344; Buller, R.M. et al. (1985) *Nature* 317(6040):813-815; McGregor, D.P. et al. (1994) *Mol. Immunol.* 31(3):219-226; and Verma, I.M. and N. Somia (1997) *Nature* 389:239-242.) The invention is not limited by the host cell employed.

For long term production of recombinant proteins in mammalian systems, stable expression of DITHP in cell lines is preferred. For example, sequences encoding DITHP can be transformed into cell lines using expression vectors which may contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Any number of selection systems may be used to recover transformed cell lines. (See, e.g., Wigler, M. et al. (1977) *Cell* 11:223-232; Lowy, I. et al. (1980) *Cell* 22:817-823; Wigler, M. et al. (1980) *Proc. Natl. Acad. Sci. USA* 77:3567-3570; Colbere-Garapin, F. et al. (1981) *J. Mol. Biol.* 150:1-14; Hartman, S.C. and R.C. Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:8047-8051; Rhodes, C.A. (1995) *Methods Mol. Biol.* 55:121-131.)

Therapeutic Uses of dithp

The dithp of the invention may be used for somatic or germline gene therapy. Gene therapy may be performed to (i) correct a genetic deficiency (e.g., in the cases of severe combined immunodeficiency (SCID)-X1 disease characterized by X-linked inheritance (Cavazzana-Calvo, M. et al. (2000) Science 288:669-672), severe combined immunodeficiency syndrome associated with an inherited adenosine deaminase (ADA) deficiency (Blaese, R.M. et al. (1995) Science 270:475-480; Bordignon, C. et al. (1995) Science 270:470-475), cystic fibrosis (Zabner, J. et al. (1993) Cell 75:207-216; Crystal, R.G. et al. (1995) Hum. Gene Therapy 6:643-666; Crystal, R.G. et al. (1995) Hum. Gene Therapy 6:667-703), thalassemias, familial hypercholesterolemia, and hemophilia resulting from Factor VIII or Factor IX deficiencies (Crystal, R.G. (1995) Science 270:404-410; Verma, I.M. and Somia, N. (1997) Nature 389:239-242)), (ii) express a conditionally lethal gene product (e.g., in the case of cancers which result from unregulated cell proliferation), or (iii) express a protein which affords protection against intracellular parasites (e.g., against human retroviruses, such as human immunodeficiency virus (HIV) (Baltimore, D. (1988) Nature 335:395-396; Poeschla, E. et al. (1996) Proc. Natl. Acad. Sci. USA. 93:11395-11399), hepatitis B or C virus (HBV, HCV); fungal parasites, such as Candida albicans and Paracoccidioides brasiliensis; and protozoan parasites such as Plasmodium falciparum and Trypanosoma cruzi). In the case where a genetic deficiency in dithp expression or regulation causes disease, the expression of dithp from an appropriate population of transduced cells may alleviate the clinical manifestations caused by the genetic deficiency.

In a further embodiment of the invention, diseases or disorders caused by deficiencies in dithp are treated by constructing mammalian expression vectors comprising dithp and introducing these vectors by mechanical means into dithp-deficient cells. Mechanical transfer technologies for use with cells in vivo or ex vitro include (i) direct DNA microinjection into individual cells, (ii) ballistic gold particle delivery, (iii) liposome-mediated transfection, (iv) receptor-mediated gene transfer, and (v) the use of DNA transposons (Morgan, R.A. and Anderson, W.F. (1993) Annu. Rev. Biochem. 62:191-217; Ivics, Z. (1997) Cell 91:501-510; Boulay, J.-L. and Ricipon, H. (1998) Curr. Opin. Biotechnol. 9:445-450).

Expression vectors that may be effective for the expression of dithp include, but are not limited to, the pCDNA 3.1, EPITAG, PRCCMV2, PREP, PVAX vectors (Invitrogen, Carlsbad CA), PCMV-SCRIPT, PCMV-TAG, PEGSH/PERV (Stratagene, La Jolla CA), and PTET-OFF, PTET-ON, PTRE2, PTRE2-LUC, PTK-HYG (Clontech, Palo Alto CA). The dithp of the invention may be expressed using (i) a constitutively active promoter, (e.g., from cytomegalovirus (CMV), Rous sarcoma virus (RSV), SV40 virus, thymidine kinase (TK), or  $\beta$ -actin genes), (ii) an inducible promoter (e.g., the tetracycline-regulated promoter (Gossen, M. and Bujard, H. (1992) Proc. Natl. Acad. Sci. U.S.A. 89:5547-5551; Gossen, M. et al., (1995) Science 268:1766-1769; Rossi, F.M.V. and Blau, H.M. (1998) Curr. Opin. Biotechnol. 9:451-456), commercially available in the T-REX plasmid

(Invitrogen)); the ecdysone-inducible promoter (available in the plasmids PVGRXR and PIND; Invitrogen); the FK506/rapamycin inducible promoter; or the RU486/mifepristone inducible promoter (Rossi, F.M.V. and Blau, H.M. supra), or (iii) a tissue-specific promoter or the native promoter of the endogenous gene encoding DITHP from a normal individual.

5 Commercially available liposome transformation kits (e.g., the PERFECT LIPID TRANSFECTION KIT, available from Invitrogen) allow one with ordinary skill in the art to deliver polynucleotides to target cells in culture and require minimal effort to optimize experimental parameters. In the alternative, transformation is performed using the calcium phosphate method (Graham, F.L. and Eb, A.J. (1973) *Virology* 52:456-467), or by electroporation (Neumann, E. et al. 10 (1982) *EMBO J.* 1:841-845). The introduction of DNA to primary cells requires modification of these standardized mammalian transfection protocols.

In another embodiment of the invention, diseases or disorders caused by genetic defects with respect to dithp expression are treated by constructing a retrovirus vector consisting of (i) the dithp of the invention under the control of an independent promoter or the retrovirus long terminal repeat (LTR) 15 promoter, (ii) appropriate RNA packaging signals, and (iii) a Rev-responsive element (RRE) along with additional retrovirus *cis*-acting RNA sequences and coding sequences required for efficient vector propagation. Retrovirus vectors (e.g., PFB and PFBNEO) are commercially available (Stratagene) and are based on published data (Riviere, I. et al. (1995) *Proc. Natl. Acad. Sci. U.S.A.* 92:6733-6737), incorporated by reference herein. The vector is propagated in an appropriate vector producing cell line 20 (VPCL) that expresses an envelope gene with a tropism for receptors on the target cells or a promiscuous envelope protein such as VSVg (Armentano, D. et al. (1987) *J. Virol.* 61:1647-1650; Bender, M.A. et al. (1987) *J. Virol.* 61:1639-1646; Adam, M.A. and Miller, A.D. (1988) *J. Virol.* 62:3802-3806; Dull, T. et al. (1998) *J. Virol.* 72:8463-8471; Zufferey, R. et al. (1998) *J. Virol.* 72:9873-9880). U.S. Patent Number 5,910,434 to Rigg ("Method for obtaining retrovirus packaging 25 cell lines producing high transducing efficiency retroviral supernatant") discloses a method for obtaining retrovirus packaging cell lines and is hereby incorporated by reference. Propagation of retrovirus vectors, transduction of a population of cells (e.g., CD4<sup>+</sup> T-cells), and the return of transduced cells to a patient are procedures well known to persons skilled in the art of gene therapy and have been well documented (Ranga, U. et al. (1997) *J. Virol.* 71:7020-7029; Baucor, G. et al. (1997) 30 *Blood* 89:2259-2267; Bonyhadi, M.L. (1997) *J. Virol.* 71:4707-4716; Ranga, U. et al. (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95:1201-1206; Su, L. (1997) *Blood* 89:2283-2290).

In the alternative, an adenovirus-based gene therapy delivery system is used to deliver dithp to cells which have one or more genetic abnormalities with respect to the expression of dithp. The construction and packaging of adenovirus-based vectors are well known to those with ordinary skill in 35 the art. Replication defective adenovirus vectors have proven to be versatile for importing genes

encoding immunoregulatory proteins into intact islets in the pancreas (Csete, M.E. et al. (1995) Transplantation 27:263-268). Potentially useful adenoviral vectors are described in U.S. Patent Number 5,707,618 to Armentano ("Adenovirus vectors for gene therapy"), hereby incorporated by reference. For adenoviral vectors, see also Antinuzzi, P.A. et al. (1999) Annu. Rev. Nutr. 19:511-544 and Verma, I.M. and Somia, N. (1997) Nature 18:389:239-242, both incorporated by reference herein.

In another alternative, a herpes-based, gene therapy delivery system is used to deliver dithp to target cells which have one or more genetic abnormalities with respect to the expression of dithp. The use of herpes simplex virus (HSV)-based vectors may be especially valuable for introducing dithp to cells of the central nervous system, for which HSV has a tropism. The construction and packaging of herpes-based vectors are well known to those with ordinary skill in the art. A replication-competent herpes simplex virus (HSV) type 1-based vector has been used to deliver a reporter gene to the eyes of primates (Liu, X. et al. (1999) Exp. Eye Res. 169:385-395). The construction of a HSV-1 virus vector has also been disclosed in detail in U.S. Patent Number 5,804,413 to DeLuca ("Herpes simplex virus strains for gene transfer"), which is hereby incorporated by reference. U.S. Patent Number 5,804,413 teaches the use of recombinant HSV d92 which consists of a genome containing at least one exogenous gene to be transferred to a cell under the control of the appropriate promoter for purposes including human gene therapy. Also taught by this patent are the construction and use of recombinant HSV strains deleted for ICP4, ICP27 and ICP22. For HSV vectors, see also Goins, W. F. et al. 1999 J. Virol. 73:519-532 and Xu, H. et al., (1994) Dev. Biol. 163:152-161, hereby incorporated by reference. The manipulation of cloned herpesvirus sequences, the generation of recombinant virus following the transfection of multiple plasmids containing different segments of the large herpesvirus genomes, the growth and propagation of herpesvirus, and the infection of cells with herpesvirus are techniques well known to those of ordinary skill in the art.

In another alternative, an alphavirus (positive, single-stranded RNA virus) vector is used to deliver dithp to target cells. The biology of the prototypic alphavirus, Semliki Forest Virus (SFV), has been studied extensively and gene transfer vectors have been based on the SFV genome (Garoff, H. and Li, K-J. (1998) Curr. Opin. Biotech. 9:464-469). During alphavirus RNA replication, a subgenomic RNA is generated that normally encodes the viral capsid proteins. This subgenomic RNA replicates to higher levels than the full-length genomic RNA, resulting in the overproduction of capsid proteins relative to the viral proteins with enzymatic activity (e.g., protease and polymerase). Similarly, inserting dithp into the alphavirus genome in place of the capsid-coding region results in the production of a large number of dithp RNAs and the synthesis of high levels of DITHP in vector transduced cells. While alphavirus infection is typically associated with cell lysis within a few days, the ability to establish a persistent infection in hamster normal kidney cells (BHK-21) with a variant of Sindbis virus (SIN) indicates that the lytic replication of alphaviruses can be altered to suit the needs of the gene

therapy application (Dryga, S.A. et al. (1997) Virology 228:74-83). The wide host range of alphaviruses will allow the introduction of DITHP into a variety of cell types. The specific transduction of a subset of cells in a population may require the sorting of cells prior to transduction. The methods of manipulating infectious cDNA clones of alphaviruses, performing alphavirus cDNA and RNA transfections, and performing alphavirus infections, are well known to those with ordinary skill in the art.

### Antibodies

Anti-DITHP antibodies may be used to analyze protein expression levels. Such antibodies include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, and Fab fragments. For descriptions of and protocols of antibody technologies, see, e.g., Pound, J.D. (1998) Immunochemical Protocols, Humana Press, Totowa, NJ.

The amino acid sequence encoded by the dithp of the Sequence Listing may be analyzed by appropriate software (e.g., LASERGENE NAVIGATOR software, DNASTAR) to determine regions of high immunogenicity. The optimal sequences for immunization are selected from the C-terminus, the N-terminus, and those intervening, hydrophilic regions of the polypeptide which are likely to be exposed to the external environment when the polypeptide is in its natural conformation. Analysis used to select appropriate epitopes is also described by Ausubel (1997, supra, Chapter 11.7). Peptides used for antibody induction do not need to have biological activity; however, they must be antigenic. Peptides used to induce specific antibodies may have an amino acid sequence consisting of at five amino acids, preferably at least 10 amino acids, and most preferably 15 amino acids. A peptide which mimics an antigenic fragment of the natural polypeptide may be fused with another protein such as keyhole limpet cyanin (KLH; Sigma, St. Louis MO) for antibody production. A peptide encompassing an antigenic region may be expressed from a dithp, synthesized as described above, or purified from human cells.

Procedures well known in the art may be used for the production of antibodies. Various hosts including mice, goats, and rabbits, may be immunized by injection with a peptide. Depending on the host species, various adjuvants may be used to increase immunological response.

In one procedure, peptides about 15 residues in length may be synthesized using an ABI 431A peptide synthesizer (PE Biosystems) using fmoc-chemistry and coupled to KLH (Sigma) by reaction with M-maleimidobenzoyl-N-hydroxysuccinimide ester (Ausubel, 1995, supra). Rabbits are immunized with the peptide-KLH complex in complete Freund's adjuvant. The resulting antisera are tested for antipeptide activity by binding the peptide to plastic, blocking with 1% bovine serum albumin (BSA), reacting with rabbit antisera, washing, and reacting with radioiodinated goat anti-rabbit IgG. Antisera with antipeptide activity are tested for anti-DITHP activity using protocols well known in the art, including ELISA, radioimmunoassay (RIA), and immunoblotting.

In another procedure, isolated and purified peptide may be used to immunize mice (about 100 µg of peptide) or rabbits (about 1 mg of peptide). Subsequently, the peptide is radioiodinated and used to screen the immunized animals' B-lymphocytes for production of anti-peptide antibodies. Positive cells are then used to produce hybridomas using standard techniques. About 20 mg of peptide is  
5 sufficient for labeling and screening several thousand clones. Hybridomas of interest are detected by screening with radioiodinated peptide to identify those fusions producing peptide-specific monoclonal antibody. In a typical protocol, wells of a multi-well plate (FAST, Becton-Dickinson, Palo Alto, CA) are coated with affinity-purified, specific rabbit-anti-mouse (or suitable anti-species IgG) antibodies at 10 mg/ml. The coated wells are blocked with 1% BSA and washed and exposed to supernatants from  
10 hybridomas. After incubation, the wells are exposed to radiolabeled peptide at 1 mg/ml.

Clones producing antibodies bind a quantity of labeled peptide that is detectable above background. Such clones are expanded and subjected to 2 cycles of cloning. Cloned hybridomas are injected into pristane-treated mice to produce ascites, and monoclonal antibody is purified from the ascitic fluid by affinity chromatography on protein A (Amersham Pharmacia Biotech). Several  
15 procedures for the production of monoclonal antibodies, including in vitro production, are described in Pound (supra). Monoclonal antibodies with anti-peptide activity are tested for anti-DITHP activity using protocols well known in the art, including ELISA, RIA, and immunoblotting.

Antibody fragments containing specific binding sites for an epitope may also be generated. For example, such fragments include, but are not limited to, the F(ab')<sub>2</sub> fragments produced by pepsin  
20 digestion of the antibody molecule, and the Fab fragments generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments. Alternatively, construction of Fab expression libraries in filamentous bacteriophage allows rapid and easy identification of monoclonal fragments with desired specificity (Pound, supra, Chaps. 45-47). Antibodies generated against polypeptide encoded by dithp can be used to purify and characterize full-length DITHP protein and its activity, binding partners, etc.

## 25 Assays Using Antibodies

Anti-DITHP antibodies may be used in assays to quantify the amount of DITHP found in a particular human cell. Such assays include methods utilizing the antibody and a label to detect expression level under normal or disease conditions. The peptides and antibodies of the invention may  
30 be used with or without modification or labeled by joining them, either covalently or noncovalently, with a reporter molecule.

Protocols for detecting and measuring protein expression using either polyclonal or monoclonal antibodies are well known in the art. Examples include ELISA, RIA, and fluorescent activated cell sorting (FACS). Such immunoassays typically involve the formation of complexes between the DITHP  
35 and its specific antibody and the measurement of such complexes. These and other assays are described



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in Pound (supra).

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

The disclosures of all patents, applications, and publications mentioned above and below, in particular U.S. Ser. No. 60/137,109, U.S. Ser. No. 60/137,337, U.S. Ser. No. 60/137,258, U.S. Ser. No. 60/137,260, U.S. Ser. No. 60/137,113, U.S. Ser. No. 60/137,161, U.S. Ser. No. 60/137,417, U.S. Ser. No. 60/137,259, U.S. Ser. No. 60/137,396, U.S. Ser. No. 60/137,114, U.S. Ser. No. 60/137,173, U.S. Ser. No. 60/137,411, U.S. Ser. No. 60/147,436, U.S. Ser. No. 60/147,549, U.S. Ser. No. 60/147,377, U.S. Ser. No. 60/147,527, U.S. Ser. No. 60/147,520, U.S. Ser. No. 60/147,536, U.S. Ser. No. 60/147,530, U.S. Ser. No. 60/147,547, U.S. Ser. No. 60/147,824, U.S. Ser. No. 60/147,541, U.S. Ser. No. 60/147,542, and U.S. Ser. No. 60/147,500, are hereby expressly incorporated by reference.

## EXAMPLES

### I. Construction of cDNA Libraries

RNA was purchased from CLONTECH Laboratories, Inc. (Palo Alto CA) or isolated from various tissues. Some tissues were homogenized and lysed in guanidinium isothiocyanate, while others were homogenized and lysed in phenol or in a suitable mixture of denaturants, such as TRIZOL (Life Technologies), a monophasic solution of phenol and guanidine isothiocyanate. The resulting lysates were centrifuged over CsCl cushions or extracted with chloroform. RNA was precipitated with either isopropanol or sodium acetate and ethanol, or by other routine methods.

Phenol extraction and precipitation of RNA were repeated as necessary to increase RNA purity. In most cases, RNA was treated with DNase. For most libraries, poly(A+) RNA was isolated using oligo d(T)-coupled paramagnetic particles (Promega Corporation (Promega), Madison WI), OLIGOTEX latex particles (QIAGEN, Inc. (QIAGEN), Valencia CA), or an OLIGOTEX mRNA purification kit (QIAGEN). Alternatively, RNA was isolated directly from tissue lysates using other RNA isolation kits, e.g., the POLY(A)PURE mRNA purification kit (Ambion, Inc., Austin TX).

In some cases, Stratagene was provided with RNA and constructed the corresponding cDNA libraries. Otherwise, cDNA was synthesized and cDNA libraries were constructed with the UNIZAP vector system (Stratagene Cloning Systems, Inc. (Stratagene), La Jolla CA) or SUPERSRIPT plasmid system (Life Technologies), using the recommended procedures or similar methods known in the art. (See, e.g., Ausubel, 1997, supra, Chapters 5.1 through 6.6.) Reverse transcription was initiated using oligo d(T) or random primers. Synthetic oligonucleotide adapters were ligated to double stranded cDNA, and the cDNA was digested with the appropriate restriction enzyme or enzymes. For

most libraries, the cDNA was size-selected (300-1000 bp) using SEPHACRYL S1000, SEPHAROSE CL2B, or SEPHAROSE CL4B column chromatography (Amersham Pharmacia Biotech) or preparative agarose gel electrophoresis. cDNAs were ligated into compatible restriction enzyme sites of the polylinker of a suitable plasmid, e.g., PBLUESCRIPT plasmid (Stratagene), pSPORT1 plasmid (Life Technologies), or pINCY (Incyte). Recombinant plasmids were transformed into competent *E. coli* cells including XL1-Blue, XL1-BlueMRF, or SOLR from Stratagene or DH5 $\alpha$ , DH10B, or ElectroMAX DH10B from Life Technologies.

## II. Isolation of cDNA Clones

Plasmids were recovered from host cells by in vivo excision using the UNIZAP vector system (Stratagene) or by cell lysis. Plasmids were purified using at least one of the following: the Magic or WIZARD Minipreps DNA purification system (Promega); the AGTC Miniprep purification kit (Edge BioSystems, Gaithersburg MD); and the QIAWELL 8, QIAWELL 8 Plus, and QIAWELL 8 Ultra plasmid purification systems or the R.E.A.L. PREP 96 plasmid purification kit (QIAGEN). Following precipitation, plasmids were resuspended in 0.1 ml of distilled water and stored, with or without lyophilization, at 4°C.

Alternatively, plasmid DNA was amplified from host cell lysates using direct link PCR in a high-throughput format. (Rao, V.B. (1994) Anal. Biochem. 216:1-14.) Host cell lysis and thermal cycling steps were carried out in a single reaction mixture. Samples were processed and stored in 384-well plates, and the concentration of amplified plasmid DNA was quantified fluorometrically using PICOGREEN dye (Molecular Probes, Inc. (Molecular Probes), Eugene OR) and a FLUOROSKAN II fluorescence scanner (Labsystems Oy, Helsinki, Finland).

## III. Sequencing and Analysis

cDNA sequencing reactions were processed using standard methods or high-throughput instrumentation such as the ABI CATALYST 800 thermal cycler (PE Biosystems) or the PTC-200 thermal cycler (MJ Research) in conjunction with the HYDRA microdispenser (Robbins Scientific Corp., Sunnyvale CA) or the MICROLAB 2200 liquid transfer system (Hamilton). cDNA sequencing reactions were prepared using reagents provided by Amersham Pharmacia Biotech or supplied in ABI sequencing kits such as the ABI PRISM BIGDYE Terminator cycle sequencing ready reaction kit (PE Biosystems). Electrophoretic separation of cDNA sequencing reactions and detection of labeled polynucleotides were carried out using the MEGABACE 1000 DNA sequencing system (Molecular Dynamics); the ABI PRISM 373 or 377 sequencing system (PE Biosystems) in conjunction with standard ABI protocols and base calling software; or other sequence analysis systems known in the art. Reading frames within the cDNA sequences were identified using standard methods (reviewed in

Ausubel, 1997, supra, Chapter 7.7). Some of the cDNA sequences were selected for extension using the techniques disclosed in Example VIII.

#### IV. Assembly and Analysis of Sequences

5 Component sequences from chromatograms were subject to PHRED analysis and assigned a quality score. The sequences having at least a required quality score were subject to various pre-processing editing pathways to eliminate, e.g., low quality 3' ends, vector and linker sequences, polyA tails, Alu repeats, mitochondrial and ribosomal sequences, bacterial contamination sequences, and sequences smaller than 50 base pairs. In particular, low-information sequences and repetitive elements  
10 (e.g., dinucleotide repeats, Alu repeats, etc.) were replaced by "n's", or masked, to prevent spurious matches.

Processed sequences were then subject to assembly procedures in which the sequences were assigned to gene bins (bins). Each sequence could only belong to one bin. Sequences in each gene bin were assembled to produce consensus sequences (templates). Subsequent new sequences were added to  
15 existing bins using BLASTn (v.1.4 WashU) and CROSSMATCH. Candidate pairs were identified as all BLAST hits having a quality score greater than or equal to 150. Alignments of at least 82% local identity were accepted into the bin. The component sequences from each bin were assembled using a version of PHRAP. Bins with several overlapping component sequences were assembled using DEEP PHRAP. The orientation (sense or antisense) of each assembled template was determined based on the  
20 number and orientation of its component sequences. Template sequences as disclosed in the sequence listing correspond to sense strand sequences (the "forward" reading frames), to the best determination. The complementary (antisense) strands are inherently disclosed herein. The component sequences which were used to assemble each template consensus sequence are listed in Table 4, along with their positions along the template nucleotide sequences.

25 Bins were compared against each other and those having local similarity of at least 82% were combined and reassembled. Reassembled bins having templates of insufficient overlap (less than 95% local identity) were re-split. Assembled templates were also subject to analysis by STITCHER/EXON MAPPER algorithms which analyze the probabilities of the presence of splice variants, alternatively spliced exons, splice junctions, differential expression of alternative spliced genes across tissue types or  
30 disease states, etc. These resulting bins were subject to several rounds of the above assembly procedures.

Once gene bins were generated based upon sequence alignments, bins were clone joined based upon clone information. If the 5' sequence of one clone was present in one bin and the 3' sequence from the same clone was present in a different bin, it was likely that the two bins actually belonged together  
35 in a single bin. The resulting combined bins underwent assembly procedures to regenerate the

consensus sequences.

The final assembled templates were subsequently annotated using the following procedure. Template sequences were analyzed using BLASTn (v2.0, NCBI) versus gbpr1 (GenBank version 116). "Hits" were defined as an exact match having from 95% local identity over 200 base pairs through 100% local identity over 100 base pairs, or a homolog match having an E-value, i.e. a probability score, of  $\leq 1 \times 10^{-8}$ . The hits were subject to frameshift FASTx versus GENPEPT (GenBank version 116). (See Table 5). In this analysis, a homolog match was defined as having an E-value of  $\leq 1 \times 10^{-8}$ . The assembly method used above was described in "System and Methods for Analyzing Biomolecular Sequences," U.S.S.N. 09/276,534, filed March 25, 1999, and the LIFESEQ Gold user manual (Incyte) both incorporated by reference herein.

Following assembly, template sequences were subjected to motif, BLAST, and functional analyses, and categorized in protein hierarchies using methods described in, e.g., "Database System Employing Protein Function Hierarchies for Viewing Biomolecular Sequence Data," U.S.S.N. 08/812,290, filed March 6, 1997; "Relational Database for Storing Biomolecule Information," U.S.S.N. 08/947,845, filed October 9, 1997; "Project-Based Full-Length Biomolecular Sequence Database," U.S.S.N. 08/811,758, filed March 6, 1997; and "Relational Database and System for Storing Information Relating to Biomolecular Sequences," U.S.S.N. 09/034,807, filed March 4, 1998, all of which are incorporated by reference herein.

The template sequences were further analyzed by translating each template in all three forward reading frames and searching each translation against the Pfam database of hidden Markov model-based protein families and domains using the HMMER software package (available to the public from Washington University School of Medicine, St. Louis MO). Regions of templates which, when translated, contain similarity to Pfam consensus sequences are reported in Table 2, along with descriptions of Pfam protein domains and families. Only those Pfam hits with an E-value of  $\leq 1 \times 10^{-3}$  are reported. (See also World Wide Web site <http://pfam.wustl.edu/> for detailed descriptions of Pfam protein domains and families.)

Additionally, the template sequences were translated in all three forward reading frames, and each translation was searched against hidden Markov models for signal peptide and transmembrane domains using the HMMER software package. Construction of hidden Markov models and their usage in sequence analysis has been described. (See, for example, Eddy, S.R. (1996) Curr. Opin. Str. Biol. 6:361-365.) Regions of templates which, when translated, contain similarity to signal peptide or transmembrane domain consensus sequences are reported in Table 3. Only those signal peptide or transmembrane hits with a cutoff score of 11 bits or greater are reported. A cutoff score of 11 bits or greater corresponds to at least about 91-94% true-positives in signal peptide prediction, and at least about 75% true-positives in transmembrane domain prediction.

The results of HMMER analysis as reported in Tables 2 and 3 may support the results of BLAST analysis as reported in Table 1 or may suggest alternative or additional properties of template-encoded polypeptides not previously uncovered by BLAST or other analyses.

- Template sequences are further analyzed using the bioinformatics tools listed in Table 5, or
- 5 using sequence analysis software known in the art such as MACDNASIS PRO software (Hitachi Software Engineering, South San Francisco CA) and LASERGENE software (DNASTAR). Template sequences may be further queried against public databases such as the GenBank rodent, mammalian, vertebrate, prokaryote, and eukaryote databases.

## 10 V. Analysis of Polynucleotide Expression

Northern analysis is a laboratory technique used to detect the presence of a transcript of a gene and involves the hybridization of a labeled nucleotide sequence to a membrane on which RNAs from a particular cell type or tissue have been bound. (See, e.g., Sambrook, supra, ch. 7; Ausubel, 1995, supra, ch. 4 and 16.)

- 15 Analogous computer techniques applying BLAST were used to search for identical or related molecules in cDNA databases such as GenBank or LIFESEQ (Incyte Pharmaceuticals). This analysis is much faster than multiple membrane-based hybridizations. In addition, the sensitivity of the computer search can be modified to determine whether any particular match is categorized as exact or similar. The basis of the search is the product score, which is defined as:

20

$$\frac{\text{BLAST Score} \times \text{Percent Identity}}{5 \times \text{minimum} \{ \text{length}(\text{Seq. 1}), \text{length}(\text{Seq. 2}) \}}$$

- The product score takes into account both the degree of similarity between two sequences and the length
- 25 of the sequence match. The product score is a normalized value between 0 and 100, and is calculated as follows: the BLAST score is multiplied by the percent nucleotide identity and the product is divided by (5 times the length of the shorter of the two sequences). The BLAST score is calculated by assigning a score of +5 for every base that matches in a high-scoring segment pair (HSP), and -4 for every mismatch. Two sequences may share more than one HSP (separated by gaps). If there is more
  - 30 than one HSP, then the pair with the highest BLAST score is used to calculate the product score. The product score represents a balance between fractional overlap and quality in a BLAST alignment. For example, a product score of 100 is produced only for 100% identity over the entire length of the shorter of the two sequences being compared. A product score of 70 is produced either by 100% identity and 70% overlap at one end, or by 88% identity and 100% overlap at the other. A product score of 50 is
  - 35 produced either by 100% identity and 50% overlap at one end, or 79% identity and 100% overlap.

## VI. Tissue Distribution Profiling

A tissue distribution profile is determined for each template by compiling the cDNA library tissue classifications of its component cDNA sequences. Each component sequence, is derived from a cDNA library constructed from a human tissue. Each human tissue is classified into one of the following categories: cardiovascular system; connective tissue; digestive system; embryonic structures; endocrine system; exocrine glands; genitalia, female; genitalia, male; germ cells; hemic and immune system; liver; musculoskeletal system; nervous system; pancreas; respiratory system; sense organs; skin; stomatognathic system; unclassified/mixed; or urinary tract. Template sequences, component sequences, and cDNA library/tissue information are found in the LIFESEQ GOLD database (Incyte Genomics, Palo Alto CA).

## VII. Transcript Image Analysis

Transcript images are generated as described in Seilhamer et al., "Comparative Gene Transcript Analysis," U.S. Patent Number 5,840,484, incorporated herein by reference.

## VIII. Extension of Polynucleotide Sequences and Isolation of a Full-length cDNA

Oligonucleotide primers designed using a dithp of the Sequence Listing are used to extend the nucleic acid sequence. One primer is synthesized to initiate 5' extension of the template, and the other primer, to initiate 3' extension of the template. The initial primers may be designed using OLIGO 4.06 software (National Biosciences, Inc. (National Biosciences), Plymouth MN), or another appropriate program, to be about 22 to 30 nucleotides in length, to have a GC content of about 50% or more, and to anneal to the target sequence at temperatures of about 68°C to about 72°C. Any stretch of nucleotides which would result in hairpin structures and primer-primer dimerizations are avoided. Selected human cDNA libraries are used to extend the sequence. If more than one extension is necessary or desired, additional or nested sets of primers are designed.

High fidelity amplification is obtained by PCR using methods well known in the art. PCR is performed in 96-well plates using the PTC-200 thermal cycler (MJ Research). The reaction mix contains DNA template, 200 nmol of each primer, reaction buffer containing  $Mg^{2+}$ ,  $(NH_4)_2SO_4$ , and  $\beta$ -mercaptoethanol, Taq DNA polymerase (Amersham Pharmacia Biotech), ELONGASE enzyme (Life Technologies), and Pfu DNA polymerase (Stratagene), with the following parameters for primer pair PCI A and PCI B: Step 1: 94°C, 3 min; Step 2: 94°C, 15 sec; Step 3: 60°C, 1 min; Step 4: 68°C, 2 min; Step 5: Steps 2, 3, and 4 repeated 20 times; Step 6: 68°C, 5 min; Step 7: storage at 4°C. In the alternative, the parameters for primer pair T7 and SK+ are as follows: Step 1: 94°C, 3 min; Step 2: 94°C, 15 sec; Step 3: 57°C, 1 min; Step 4: 68°C, 2 min; Step 5: Steps 2, 3, and 4 repeated 20 times;

Step 6: 68°C, 5 min; Step 7: storage at 4°C.

The concentration of DNA in each well is determined by dispensing 100 µl PICOGREEN quantitation reagent (0.25% (v/v); Molecular Probes) dissolved in 1X Tris-EDTA (TE) and 0.5 µl of undiluted PCR product into each well of an opaque fluorimeter plate (Corning Incorporated (Corning),  
5 Corning NY), allowing the DNA to bind to the reagent. The plate is scanned in a FLUOROSKAN II (Labsystems Oy) to measure the fluorescence of the sample and to quantify the concentration of DNA. A 5 µl to 10 µl aliquot of the reaction mixture is analyzed by electrophoresis on a 1 % agarose mini-gel to determine which reactions are successful in extending the sequence.

The extended nucleotides are desalted and concentrated, transferred to 384-well plates,  
10 digested with CviJI cholera virus endonuclease (Molecular Biology Research, Madison WI), and sonicated or sheared prior to religation into pUC 18 vector (Amersham Pharmacia Biotech). For shotgun sequencing, the digested nucleotides are separated on low concentration (0.6 to 0.8%) agarose gels, fragments are excised, and agar digested with AGAR ACE (Promega). Extended clones are religated using T4 ligase (New England Biolabs, Inc., Beverly MA) into pUC 18 vector (Amersham  
15 Pharmacia Biotech), treated with Pfu DNA polymerase (Stratagene) to fill-in restriction site overhangs, and transfected into competent E. coli cells. Transformed cells are selected on antibiotic-containing media, individual colonies are picked and cultured overnight at 37°C in 384-well plates in LB/2x carbenicillin liquid media.

The cells are lysed, and DNA is amplified by PCR using Taq DNA polymerase (Amersham  
20 Pharmacia Biotech) and Pfu DNA polymerase (Stratagene) with the following parameters: Step 1: 94°C, 3 min; Step 2: 94°C, 15 sec; Step 3: 60°C, 1 min; Step 4: 72°C, 2 min; Step 5: steps 2, 3, and 4 repeated 29 times; Step 6: 72°C, 5 min; Step 7: storage at 4°C. DNA is quantified by PICOGREEN reagent (Molecular Probes) as described above. Samples with low DNA recoveries are reamplified using the same conditions as described above. Samples are diluted with 20% dimethylsulfoxide (1:2,  
25 v/v), and sequenced using DYENAMIC energy transfer sequencing primers and the DYENAMIC DIRECT kit (Amersham Pharmacia Biotech) or the ABI PRISM BIGDYE Terminator cycle sequencing ready reaction kit (PE Biosystems).

In like manner, the dithp is used to obtain regulatory sequences (promoters, introns, and enhancers) using the procedure above, oligonucleotides designed for such extension, and an appropriate  
30 genomic library.

## IX. Labeling of Probes and Southern Hybridization Analyses

Hybridization probes derived from the dithp of the Sequence Listing are employed for screening cDNAs, mRNAs, or genomic DNA. The labeling of probe nucleotides between 100 and  
35 1000 nucleotides in length is specifically described, but essentially the same procedure may be used

with larger cDNA fragments. Probe sequences are labeled at room temperature for 30 minutes using a T4 polynucleotide kinase,  $\gamma^{32}\text{P}$ -ATP, and 0.5X One-Phor-All Plus (Amersham Pharmacia Biotech) buffer and purified using a ProbeQuant G-50 Microcolumn (Amersham Pharmacia Biotech). The probe mixture is diluted to  $10^7$  dpm/ $\mu\text{g}/\text{ml}$  hybridization buffer and used in a typical membrane-based hybridization analysis.

The DNA is digested with a restriction endonuclease such as Eco RV and is electrophoresed through a 0.7% agarose gel. The DNA fragments are transferred from the agarose to nylon membrane (NYTRAN Plus, Schleicher & Schuell, Inc., Keene NH) using procedures specified by the manufacturer of the membrane. Prehybridization is carried out for three or more hours at  $68^\circ\text{C}$ , and hybridization is carried out overnight at  $68^\circ\text{C}$ . To remove non-specific signals, blots are sequentially washed at room temperature under increasingly stringent conditions, up to 0.1x saline sodium citrate (SSC) and 0.5% sodium dodecyl sulfate. After the blots are placed in a PHOSPHORIMAGER cassette (Molecular Dynamics) or are exposed to autoradiography film, hybridization patterns of standard and experimental lanes are compared. Essentially the same procedure is employed when screening RNA.

## X. Chromosome Mapping of dithp

The cDNA sequences which were used to assemble SEQ ID NO:1-52 are compared with sequences from the Incyte LIFESEQ database and public domain databases using BLAST and other implementations of the Smith-Waterman algorithm. Sequences from these databases that match SEQ ID NO:1-52 are assembled into clusters of contiguous and overlapping sequences using assembly algorithms such as PHRAP (Table 5). Radiation hybrid and genetic mapping data available from public resources such as the Stanford Human Genome Center (SHGC), Whitehead Institute for Genome Research (WIGR), and Génethon are used to determine if any of the clustered sequences have been previously mapped. Inclusion of a mapped sequence in a cluster will result in the assignment of all sequences of that cluster, including its particular SEQ ID NO., to that map location. The genetic map locations of SEQ ID NO:1-52 are described as ranges, or intervals, of human chromosomes. The map position of an interval, in centiMorgans, is measured relative to the terminus of the chromosome's p-arm. (The centiMorgan (cM) is a unit of measurement based on recombination frequencies between chromosomal markers. On average, 1 cM is roughly equivalent to 1 megabase (Mb) of DNA in humans, although this can vary widely due to hot and cold spots of recombination.) The cM distances are based on genetic markers mapped by Génethon which provide boundaries for radiation hybrid markers whose sequences were included in each of the clusters.

## XI. Microarray Analysis

### Probe Preparation from Tissue or Cell Samples



Total RNA is isolated from tissue samples using the guanidinium thiocyanate method and polyA<sup>+</sup> RNA is purified using the oligo (dT) cellulose method. Each polyA<sup>+</sup> RNA sample is reverse transcribed using MMLV reverse-transcriptase, 0.05 pg/ $\mu$ l oligo-dT primer (21mer), 1X first strand buffer, 0.03 units/ $\mu$ l RNase inhibitor, 500  $\mu$ M dATP, 500  $\mu$ M dGTP, 500  $\mu$ M dTTP, 40  $\mu$ M dCTP, 40  $\mu$ M dCTP-Cy3 (BDS) or dCTP-Cy5 (Amersham Pharmacia Biotech). The reverse transcription reaction is performed in a 25 ml volume containing 200 ng polyA<sup>+</sup> RNA with GEMBRIGHT kits (Incyte). Specific control polyA<sup>+</sup> RNAs are synthesized by *in vitro* transcription from non-coding yeast genomic DNA (W. Lei, unpublished). As quantitative controls, the control mRNAs at 0.002 ng, 0.02 ng, 0.2 ng, and 2 ng are diluted into reverse transcription reaction at ratios of 1:100,000, 1:10,000, 1:1,000, 1:100 (w/w) to sample mRNA respectively. The control mRNAs are diluted into reverse transcription reaction at ratios of 1:3, 3:1, 1:10, 10:1, 1:25, 25:1 (w/w) to sample mRNA differential expression patterns. After incubation at 37°C for 2 hr, each reaction sample (one with Cy3 and another with Cy5 labeling) is treated with 2.5 ml of 0.5M sodium hydroxide and incubated for 20 minutes at 85°C to stop the reaction and degrade the RNA. Probes are purified using two successive CHROMA SPIN 30 gel filtration spin columns (CLONTECH Laboratories, Inc. (CLONTECH), Palo Alto CA) and after combining, both reaction samples are ethanol precipitated using 1 ml of glycogen (1 mg/ml), 60 ml sodium acetate, and 300 ml of 100% ethanol. The probe is then dried to completion using a SpeedVAC (Savant Instruments Inc., Holbrook NY) and resuspended in 14  $\mu$ l 5X SSC/0.2% SDS.

#### Microarray Preparation

Sequences of the present invention are used to generate array elements. Each array element is amplified from bacterial cells containing vectors with cloned cDNA inserts. PCR amplification uses primers complementary to the vector sequences flanking the cDNA insert. Array elements are amplified in thirty cycles of PCR from an initial quantity of 1-2 ng to a final quantity greater than 5  $\mu$ g. Amplified array elements are then purified using SEPHACRYL-400 (Amersham Pharmacia Biotech).

Purified array elements are immobilized on polymer-coated glass slides. Glass microscope slides (Corning) are cleaned by ultrasound in 0.1% SDS and acetone, with extensive distilled water washes between and after treatments. Glass slides are etched in 4% hydrofluoric acid (VWR Scientific Products Corporation (VWR), West Chester, PA), washed extensively in distilled water, and coated with 0.05% aminopropyl silane (Sigma) in 95% ethanol. Coated slides are cured in a 110°C oven.

Array elements are applied to the coated glass substrate using a procedure described in US Patent No. 5,807,522, incorporated herein by reference. 1  $\mu$ l of the array element DNA, at an average concentration of 100 ng/ $\mu$ l, is loaded into the open capillary printing element by a high-speed robotic apparatus. The apparatus then deposits about 5 nl of array element sample per slide.

Microarrays are UV-crosslinked using a STRATALINKER UV-crosslinker (Stratagene). Microarrays are washed at room temperature once in 0.2% SDS and three times in distilled water. Non-specific binding sites are blocked by incubation of microarrays in 0.2% casein in phosphate buffered saline (PBS) (Tropix, Inc., Bedford, MA) for 30 minutes at 60°C followed by washes in 0.2% SDS and distilled water as before.

#### Hybridization

Hybridization reactions contain 9 µl of probe mixture consisting of 0.2 µg each of Cy3 and Cy5 labeled cDNA synthesis products in 5X SSC, 0.2% SDS hybridization buffer. The probe mixture is heated to 65°C for 5 minutes and is aliquoted onto the microarray surface and covered with an 1.8 cm<sup>2</sup> coverslip. The arrays are transferred to a waterproof chamber having a cavity just slightly larger than a microscope slide. The chamber is kept at 100% humidity internally by the addition of 140 µl of 5x SSC in a corner of the chamber. The chamber containing the arrays is incubated for about 6.5 hours at 60°C. The arrays are washed for 10 min at 45°C in a first wash buffer (1X SSC, 0.1% SDS), three times for 10 minutes each at 45°C in a second wash buffer (0.1X SSC), and dried.

#### Detection

Reporter-labeled hybridization complexes are detected with a microscope equipped with an Innova 70 mixed gas 10 W laser (Coherent, Inc., Santa Clara CA) capable of generating spectral lines at 488 nm for excitation of Cy3 and at 632 nm for excitation of Cy5. The excitation laser light is focused on the array using a 20X microscope objective (Nikon, Inc., Melville NY). The slide containing the array is placed on a computer-controlled X-Y stage on the microscope and raster-scanned past the objective. The 1.8 cm x 1.8 cm array used in the present example is scanned with a resolution of 20 micrometers.

In two separate scans, a mixed gas multiline laser excites the two fluorophores sequentially. Emitted light is split, based on wavelength, into two photomultiplier tube detectors (PMT R1477, Hamamatsu Photonics Systems, Bridgewater NJ) corresponding to the two fluorophores. Appropriate filters positioned between the array and the photomultiplier tubes are used to filter the signals. The emission maxima of the fluorophores used are 565 nm for Cy3 and 650 nm for Cy5. Each array is typically scanned twice, one scan per fluorophore using the appropriate filters at the laser source, although the apparatus is capable of recording the spectra from both fluorophores simultaneously.

The sensitivity of the scans is typically calibrated using the signal intensity generated by a cDNA control species added to the probe mix at a known concentration. A specific location on the array contains a complementary DNA sequence, allowing the intensity of the signal at that location to be correlated with a weight ratio of hybridizing species of 1:100,000. When two probes from different

sources (e.g., representing test and control cells), each labeled with a different fluorophore, are hybridized to a single array for the purpose of identifying genes that are differentially expressed, the calibration is done by labeling samples of the calibrating cDNA with the two fluorophores and adding identical amounts of each to the hybridization mixture.

5 The output of the photomultiplier tube is digitized using a 12-bit RTI-835H analog-to-digital (A/D) conversion board (Analog Devices, Inc., Norwood, MA) installed in an IBM-compatible PC computer. The digitized data are displayed as an image where the signal intensity is mapped using a linear 20-color transformation to a pseudocolor scale ranging from blue (low signal) to red (high signal). The data is also analyzed quantitatively. Where two different fluorophores are excited and  
10 measured simultaneously, the data are first corrected for optical crosstalk (due to overlapping emission spectra) between the fluorophores using each fluorophore's emission spectrum.

A grid is superimposed over the fluorescence signal image such that the signal from each spot is centered in each element of the grid. The fluorescence signal within each element is then integrated to obtain a numerical value corresponding to the average intensity of the signal. The software used for  
15 signal analysis is the GEMTOOLS gene expression analysis program (Incyte).

## XII. Complementary Nucleic Acids

Sequences complementary to the dithp are used to detect, decrease, or inhibit expression of the naturally occurring nucleotide. The use of oligonucleotides comprising from about 15 to 30 base pairs  
20 is typical in the art. However, smaller or larger sequence fragments can also be used. Appropriate oligonucleotides are designed from the dithp using OLIGO 4.06 software (National Biosciences) or other appropriate programs and are synthesized using methods standard in the art or ordered from a commercial supplier. To inhibit transcription, a complementary oligonucleotide is designed from the most unique 5' sequence and used to prevent transcription factor binding to the promoter sequence. To  
25 inhibit translation, a complementary oligonucleotide is designed to prevent ribosomal binding and processing of the transcript.

## XIII. Expression of DITHP

Expression and purification of DITHP is accomplished using bacterial or virus-based  
30 expression systems. For expression of DITHP in bacteria, cDNA is subcloned into an appropriate vector containing an antibiotic resistance gene and an inducible promoter that directs high levels of cDNA transcription. Examples of such promoters include, but are not limited to, the *trp-lac* (*tac*) hybrid promoter and the T5 or T7 bacteriophage promoter in conjunction with the *lac* operator regulatory element. Recombinant vectors are transformed into suitable bacterial hosts, e.g.,  
35 BL21(DE3). Antibiotic resistant bacteria express DITHP upon induction with isopropyl beta-D-

thiogalactopyranoside (IPTG). Expression of DITHP in eukaryotic cells is achieved by infecting insect or mammalian cell lines with recombinant *Autographica californica* nuclear polyhedrosis virus (AcMNPV), commonly known as baculovirus. The nonessential polyhedrin gene of baculovirus is replaced with cDNA encoding DITHP by either homologous recombination or bacterial-mediated transposition involving transfer plasmid intermediates. Viral infectivity is maintained and the strong polyhedrin promoter drives high levels of cDNA transcription. Recombinant baculovirus is used to infect *Spodoptera frugiperda* (Sf9) insect cells in most cases, or human hepatocytes, in some cases. Infection of the latter requires additional genetic modifications to baculovirus. (See e.g., Engelhard, supra; and Sandig, supra.)

In most expression systems, DITHP is synthesized as a fusion protein with, e.g., glutathione S-transferase (GST) or a peptide epitope tag, such as FLAG or 6-His, permitting rapid, single-step, affinity-based purification of recombinant fusion protein from crude cell lysates. GST, a 26-kilodalton enzyme from *Schistosoma japonicum*, enables the purification of fusion proteins on immobilized glutathione under conditions that maintain protein activity and antigenicity (Amersham Pharmacia Biotech). Following purification, the GST moiety can be proteolytically cleaved from DITHP at specifically engineered sites. FLAG, an 8-amino acid peptide, enables immunoaffinity purification using commercially available monoclonal and polyclonal anti-FLAG antibodies (Eastman Kodak Company, Rochester NY). 6-His, a stretch of six consecutive histidine residues, enables purification on metal-chelate resins (QIAGEN). Methods for protein expression and purification are discussed in Ausubel (1995, supra, Chapters 10 and 16). Purified DITHP obtained by these methods can be used directly in the following activity assay.

#### XIV. Demonstration of DITHP Activity

DITHP activity is demonstrated through a variety of specific assays, some of which are outlined below.

Oxidoreductase activity of DITHP is measured by the increase in extinction coefficient of NAD(P)H coenzyme at 340 nm for the measurement of oxidation activity, or the decrease in extinction coefficient of NAD(P)H coenzyme at 340 nm for the measurement of reduction activity (Dalziel, K. (1963) J. Biol. Chem. 238:2850-2858). One of three substrates may be used: Asn-βGal, biocytidine, or ubiquinone-10. The respective subunits of the enzyme reaction, for example, cytochrome c<sub>1</sub>-b oxidoreductase and cytochrome c, are reconstituted. The reaction mixture contains a) 1-2 mg/ml DITHP; and b) 15 mM substrate, 2.4 mM NAD(P)<sup>+</sup> in 0.1 M phosphate buffer, pH 7.1 (oxidation reaction), or 2.0 mM NAD(P)H, in 0.1 M Na<sub>2</sub>HPO<sub>4</sub> buffer, pH 7.4 (reduction reaction); in a total volume of 0.1 ml. Changes in absorbance at 340 nm (A<sub>340</sub>) are measured at 23.5° C using a recording spectrophotometer (Shimadzu Scientific Instruments, Inc., Pleasanton CA). The amount of NAD(P)H

is stoichiometrically equivalent to the amount of substrate initially present, and the change in  $A_{340}$  is a direct measure of the amount of NAD(P)H produced;  $\Delta A_{340} = 6620[\text{NADH}]$ . Oxidoreductase activity of DITHP activity is proportional to the amount of NAD(P)H present in the assay.

Transferase activity of DITHP is measured through assays such as a methyl transferase assay  
 5 in which the transfer of radiolabeled methyl groups between a donor substrate and an acceptor substrate is measured (Bokar, J.A. et al. (1994) J. Biol. Chem. 269:17697-17704). Reaction mixtures (50  $\mu\text{l}$  final volume) contain 15 mM HEPES, pH 7.9, 1.5 mM  $\text{MgCl}_2$ , 10 mM dithiothreitol, 3% polyvinylalcohol, 1.5  $\mu\text{Ci}$  [ $\text{methyl-}^3\text{H}$ ]AdoMet (0.375  $\mu\text{M}$  AdoMet) (DuPont-NEN), 0.6  $\mu\text{g}$  DITHP, and acceptor substrate (0.4  $\mu\text{g}$  [ $^{35}\text{S}$ ]RNA or 6-mercaptapurine (6-MP) to 1 mM final concentration).  
 10 Reaction mixtures are incubated at 30°C for 30 minutes, then 65°C for 5 minutes. The products are separated by chromatography or electrophoresis and the level of methyl transferase activity is determined by quantification of  $\text{methyl-}^3\text{H}$  recovery.

DITHP hydrolase activity is measured by the hydrolysis of appropriate synthetic peptide substrates conjugated with various chromogenic molecules in which the degree of hydrolysis is  
 15 quantified by spectrophotometric (or fluorometric) absorption of the released chromophore. (Beynon, R.J. and J.S. Bond (1994) Proteolytic Enzymes: A Practical Approach, Oxford University Press, New York NY, pp. 25-55) Peptide substrates are designed according to the category of protease activity as endopeptidase (serine, cysteine, aspartic proteases), aminopeptidase (leucine aminopeptidase), or carboxypeptidase (Carboxypeptidase A and B, procollagen C-proteinase).

DITHP isomerase activity such as peptidyl prolyl *cis/trans* isomerase activity can be assayed  
 20 by an enzyme assay described by Rahfeld, J.U., et al. (1994) (FEBS Lett. 352: 180-184). The assay is performed at 10°C in 35 mM HEPES buffer, pH 7.8, containing chymotrypsin (0.5 mg/ml) and DITHP at a variety of concentrations. Under these assay conditions, the substrate, Suc-Ala-Xaa-Pro-Phe-4-NA, is in equilibrium with respect to the prolyl bond, with 80-95% in *trans* and 5-20% in *cis* conformation. An aliquot (2  $\mu\text{l}$ ) of the substrate dissolved in dimethyl sulfoxide (10 mg/ml) is added  
 25 to the reaction mixture described above. Only the *cis* isomer of the substrate is a substrate for cleavage by chymotrypsin. Thus, as the substrate is isomerized by DITHP, the product is cleaved by chymotrypsin to produce 4-nitroanilide, which is detected by its absorbance at 390 nm. 4-Nitroanilide appears in a time-dependent and a DITHP concentration-dependent manner.

An assay for DITHP activity associated with growth and development measures cell  
 30 proliferation as the amount of newly initiated DNA synthesis in Swiss mouse 3T3 cells. A plasmid containing polynucleotides encoding DITHP is transfected into quiescent 3T3 cultured cells using methods well known in the art. The transiently transfected cells are then incubated in the presence of [ $^3\text{H}$ ]thymidine, a radioactive DNA precursor. Where applicable, varying amounts of DITHP ligand are  
 35 added to the transfected cells. Incorporation of [ $^3\text{H}$ ]thymidine into acid-precipitable DNA is measured

over an appropriate time interval, and the amount incorporated is directly proportional to the amount of newly synthesized DNA.

Growth factor activity of DITHP is measured by the stimulation of DNA synthesis in Swiss mouse 3T3 cells (McKay, I. and I. Leigh, eds. (1993) Growth Factors: A Practical Approach, Oxford University Press, New York NY). Initiation of DNA synthesis indicates the cells' entry into the mitotic cycle and their commitment to undergo later division. 3T3 cells are competent to respond to most growth factors, not only those that are mitogenic, but also those that are involved in embryonic induction. This competence is possible because the in vivo specificity demonstrated by some growth factors is not necessarily inherent but is determined by the responding tissue. In this assay, varying amounts of DITHP are added to quiescent 3T3 cultured cells in the presence of [<sup>3</sup>H]thymidine, a radioactive DNA precursor. DITHP for this assay can be obtained by recombinant means or from biochemical preparations. Incorporation of [<sup>3</sup>H]thymidine into acid-precipitable DNA is measured over an appropriate time interval, and the amount incorporated is directly proportional to the amount of newly synthesized DNA. A linear dose-response curve over at least a hundred-fold DITHP concentration range is indicative of growth factor activity. One unit of activity per milliliter is defined as the concentration of DITHP producing a 50% response level, where 100% represents maximal incorporation of [<sup>3</sup>H]thymidine into acid-precipitable DNA.

Alternatively, an assay for cytokine activity of DITHP measures the proliferation of leukocytes. In this assay, the amount of tritiated thymidine incorporated into newly synthesized DNA is used to estimate proliferative activity. Varying amounts of DITHP are added to cultured leukocytes, such as granulocytes, monocytes, or lymphocytes, in the presence of [<sup>3</sup>H]thymidine, a radioactive DNA precursor. DITHP for this assay can be obtained by recombinant means or from biochemical preparations. Incorporation of [<sup>3</sup>H]thymidine into acid-precipitable DNA is measured over an appropriate time interval, and the amount incorporated is directly proportional to the amount of newly synthesized DNA. A linear dose-response curve over at least a hundred-fold DITHP concentration range is indicative of DITHP activity. One unit of activity per milliliter is conventionally defined as the concentration of DITHP producing a 50% response level, where 100% represents maximal incorporation of [<sup>3</sup>H]thymidine into acid-precipitable DNA.

An alternative assay for DITHP cytokine activity utilizes a Boyden micro chamber (Neuroprobe, Cabin John MD) to measure leukocyte chemotaxis (Vicari, supra). In this assay, about 10<sup>5</sup> migratory cells such as macrophages or monocytes are placed in cell culture media in the upper compartment of the chamber. Varying dilutions of DITHP are placed in the lower compartment. The two compartments are separated by a 5 or 8 micron pore polycarbonate filter (Nucleopore, Pleasanton CA). After incubation at 37°C for 80 to 120 minutes, the filters are fixed in methanol and stained with appropriate labeling agents. Cells which migrate to the other side of the filter are counted using

standard microscopy. The chemotactic index is calculated by dividing the number of migratory cells counted when DITHP is present in the lower compartment by the number of migratory cells counted when only media is present in the lower compartment. The chemotactic index is proportional to the activity of DITHP.

Alternatively, cell lines or tissues transformed with a vector containing dithp can be assayed for DITHP activity by immunoblotting. Cells are denatured in SDS in the presence of  $\beta$ -mercaptoethanol, nucleic acids removed by ethanol precipitation, and proteins purified by acetone precipitation. Pellets are resuspended in 20 mM tris buffer at pH 7.5 and incubated with Protein G-Sepharose pre-coated with an antibody specific for DITHP. After washing, the Sepharose beads are boiled in electrophoresis sample buffer, and the eluted proteins subjected to SDS-PAGE. The SDS-PAGE is transferred to a nitrocellulose membrane for immunoblotting, and the DITHP activity is assessed by visualizing and quantifying bands on the blot using the antibody specific for DITHP as the primary antibody and  $^{125}$ I-labeled IgG specific for the primary antibody as the secondary antibody.

DITHP kinase activity is measured by phosphorylation of a protein substrate using  $\gamma$ -labeled [ $^{32}$ P]-ATP and quantitation of the incorporated radioactivity using a radioisotope counter. DITHP is incubated with the protein substrate, [ $^{32}$ P]-ATP, and an appropriate kinase buffer. The [ $^{32}$ P] incorporated into the product is separated from free [ $^{32}$ P]-ATP by electrophoresis and the incorporated [ $^{32}$ P] is counted. The amount of [ $^{32}$ P] recovered is proportional to the kinase activity of DITHP in the assay. A determination of the specific amino acid residue phosphorylated is made by phosphoamino acid analysis of the hydrolyzed protein.

In the alternative, DITHP activity is measured by the increase in cell proliferation resulting from transformation of a mammalian cell line such as COS7, HeLa or CHO with an eukaryotic expression vector encoding DITHP. Eukaryotic expression vectors are commercially available, and the techniques to introduce them into cells are well known to those skilled in the art. The cells are incubated for 48-72 hours after transformation under conditions appropriate for the cell line to allow expression of DITHP. Phase microscopy is then used to compare the mitotic index of transformed versus control cells. An increase in the mitotic index indicates DITHP activity.

In a further alternative, an assay for DITHP signaling activity is based upon the ability of GPCR family proteins to modulate G protein-activated second messenger signal transduction pathways (e.g., cAMP; Gaudin, P. et al. (1998) J. Biol. Chem. 273:4990-4996). A plasmid encoding full length DITHP is transfected into a mammalian cell line (e.g., Chinese hamster ovary (CHO) or human embryonic kidney (HEK-293) cell lines) using methods well-known in the art. Transfected cells are grown in 12-well trays in culture medium for 48 hours, then the culture medium is discarded, and the attached cells are gently washed with PBS. The cells are then incubated in culture medium with or without ligand for 30 minutes, then the medium is removed and cells lysed by treatment with

1 M perchloric acid. The cAMP levels in the lysate are measured by radioimmunoassay using methods well-known in the art. Changes in the levels of cAMP in the lysate from cells exposed to ligand compared to those without ligand are proportional to the amount of DITHP present in the transfected cells.

Alternatively, an assay for DITHP protein phosphatase activity measures the hydrolysis of P-nitrophenyl phosphate (PNPP). DITHP is incubated together with PNPP in HEPES buffer pH 7.5, in the presence of 0.1%  $\beta$ -mercaptoethanol at 37°C for 60 min. The reaction is stopped by the addition of 6 ml of 10 N NaOH, and the increase in light absorbance of the reaction mixture at 410 nm resulting from the hydrolysis of PNPP is measured using a spectrophotometer. The increase in light absorbance is proportional to the phosphatase activity of DITHP in the assay (Diamond, R.H. et al (1994) Mol Cell Biol 14:3752-3762).

An alternative assay measures DITHP-mediated G-protein signaling activity by monitoring the mobilization of  $Ca^{++}$  as an indicator of the signal transduction pathway stimulation. (See, e.g., Grynkiewicz, G. et al. (1985) J. Biol. Chem. 260:3440; McCall, S. et al. (1993) J. Immunol. 150:4550-4555; and Ausset, C. et al. (1988) J. Immunol. 140:215-220). The assay requires preloading neutrophils or T cells with a fluorescent dye such as FURA-2 or BCECF (Universal Imaging Corp, Westchester PA) whose emission characteristics are altered by  $Ca^{++}$  binding. When the cells are exposed to one or more activating stimuli artificially (e.g., anti-CD3 antibody ligation of the T cell receptor) or physiologically (e.g., by allogeneic stimulation),  $Ca^{++}$  flux takes place. This flux can be observed and quantified by assaying the cells in a fluorometer or fluorescent activated cell sorter. Measurements of  $Ca^{++}$  flux are compared between cells in their normal state and those transfected with DITHP. Increased  $Ca^{++}$  mobilization attributable to increased DITHP concentration is proportional to DITHP activity.

DITHP transport activity is assayed by measuring uptake of labeled substrates into Xenopus laevis oocytes. Oocytes at stages V and VI are injected with DITHP mRNA (10 ng per oocyte) and incubated for 3 days at 18°C in OR2 medium (82.5mM NaCl, 2.5 mM KCl, 1mM  $CaCl_2$ , 1mM  $MgCl_2$ , 1mM  $Na_2HPO_4$ , 5 mM Hepes, 3.8 mM NaOH, 50 $\mu$ g/ml gentamycin, pH 7.8) to allow expression of DITHP protein. Oocytes are then transferred to standard uptake medium (100mM NaCl, 2 mM KCl, 1mM  $CaCl_2$ , 1mM  $MgCl_2$ , 10 mM Hepes/Tris pH 7.5). Uptake of various substrates (e.g., amino acids, sugars, drugs, ions, and neurotransmitters) is initiated by adding labeled substrate (e.g. radiolabeled with  $^3H$ , fluorescently labeled with rhodamine, etc.) to the oocytes. After incubating for 30 minutes, uptake is terminated by washing the oocytes three times in  $Na^+$ -free medium, measuring the incorporated label, and comparing with controls. DITHP transport activity is proportional to the level of internalized labeled substrate.

DITHP transferase activity is demonstrated by a test for galactosyltransferase activity. This



can be determined by measuring the transfer of radiolabeled galactose from UDP-galactose to a GlcNAc-terminated oligosaccharide chain (Kolbinger, F. et al. (1998) *J. Biol. Chem.* 273:58-65). The sample is incubated with 14  $\mu$ l of assay stock solution (180 mM sodium cacodylate, pH 6.5, 1 mg/ml bovine serum albumin, 0.26 mM UDP-galactose, 2  $\mu$ l of UDP-[<sup>3</sup>H]galactose), 1  $\mu$ l of MnCl<sub>2</sub> (500 mM), and 2.5  $\mu$ l of GlcNAc $\beta$ O-(CH<sub>2</sub>)<sub>4</sub>-CO<sub>2</sub>Me (37 mg/ml in dimethyl sulfoxide) for 60 minutes at 37°C. The reaction is quenched by the addition of 1 ml of water and loaded on a C18 Sep-Pak cartridge (Waters), and the column is washed twice with 5 ml of water to remove unreacted UDP-[<sup>3</sup>H]galactose. The [<sup>3</sup>H]galactosylated GlcNAc $\beta$ O-(CH<sub>2</sub>)<sub>4</sub>-CO<sub>2</sub>Me remains bound to the column during the water washes and is eluted with 5 ml of methanol. Radioactivity in the eluted material is measured by liquid scintillation counting and is proportional to galactosyltransferase activity in the starting sample.

In the alternative, DITHP induction by heat or toxins may be demonstrated using primary cultures of human fibroblasts or human cell lines such as CCL-13, HEK293, or HEP G2 (ATCC). To heat induce DITHP expression, aliquots of cells are incubated at 42 °C for 15, 30, or 60 minutes. Control aliquots are incubated at 37 °C for the same time periods. To induce DITHP expression by toxins, aliquots of cells are treated with 100  $\mu$ M arsenite or 20 mM azetidine-2-carboxylic acid for 0, 3, 6, or 12 hours. After exposure to heat, arsenite, or the amino acid analogue, samples of the treated cells are harvested and cell lysates prepared for analysis by western blot. Cells are lysed in lysis buffer containing 1% Nonidet P-40, 0.15 M NaCl, 50 mM Tris-HCl, 5 mM EDTA, 2 mM N-ethylmaleimide, 2 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, and 1 mg/ml pepstatin. Twenty micrograms of the cell lysate is separated on an 8% SDS-PAGE gel and transferred to a membrane. After blocking with 5% nonfat dry milk/phosphate-buffered saline for 1 h, the membrane is incubated overnight at 4°C or at room temperature for 2-4 hours with a 1:1000 dilution of anti-DITHP serum in 2% nonfat dry milk/phosphate-buffered saline. The membrane is then washed and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG in 2% dry milk/phosphate-buffered saline. After washing with 0.1% Tween 20 in phosphate-buffered saline, the DITHP protein is detected and compared to controls using chemiluminescence.

Alternatively, DITHP protease activity is measured by the hydrolysis of appropriate synthetic peptide substrates conjugated with various chromogenic molecules in which the degree of hydrolysis is quantified by spectrophotometric (or fluorometric) absorption of the released chromophore (Beynon, R.J. and J.S. Bond (1994) Proteolytic Enzymes: A Practical Approach, Oxford University Press, New York, NY, pp.25-55). Peptide substrates are designed according to the category of protease activity as endopeptidase (serine, cysteine, aspartic proteases, or metalloproteases), aminopeptidase (leucine aminopeptidase), or carboxypeptidase (carboxypeptidases A and B, procollagen C-proteinase). Commonly used chromogens are 2-naphthylamine, 4-nitroaniline, and

furylacrylic acid. Assays are performed at ambient temperature and contain an aliquot of the enzyme and the appropriate substrate in a suitable buffer. Reactions are carried out in an optical cuvette, and the increase/decrease in absorbance of the chromogen released during hydrolysis of the peptide substrate is measured. The change in absorbance is proportional to the DITHP protease activity in the assay.

In the alternative, an assay for DITHP protease activity takes advantage of fluorescence resonance energy transfer (FRET) that occurs when one donor and one acceptor fluorophore with an appropriate spectral overlap are in close proximity. A flexible peptide linker containing a cleavage site specific for PRTS is fused between a red-shifted variant (RSGFP4) and a blue variant (BFP5) of Green Fluorescent Protein. This fusion protein has spectral properties that suggest energy transfer is occurring from BFP5 to RSGFP4. When the fusion protein is incubated with DITHP, the substrate is cleaved, and the two fluorescent proteins dissociate. This is accompanied by a marked decrease in energy transfer which is quantified by comparing the emission spectra before and after the addition of DITHP (Mitra, R.D. et al (1996) Gene 173:13-17). This assay can also be performed in living cells. In this case the fluorescent substrate protein is expressed constitutively in cells and DITHP is introduced on an inducible vector so that FRET can be monitored in the presence and absence of DITHP (Sagot, I. et al (1999) FEBS Lett. 447:53-57).

A method to determine the nucleic acid binding activity of DITHP involves a polyacrylamide gel mobility-shift assay. In preparation for this assay, DITHP is expressed by transforming a mammalian cell line such as COS7, HeLa or CHO with a eukaryotic expression vector containing DITHP cDNA. The cells are incubated for 48-72 hours after transformation under conditions appropriate for the cell line to allow expression and accumulation of DITHP. Extracts containing solubilized proteins can be prepared from cells expressing DITHP by methods well known in the art. Portions of the extract containing DITHP are added to [<sup>32</sup>P]-labeled RNA or DNA. Radioactive nucleic acid can be synthesized in vitro by techniques well known in the art. The mixtures are incubated at 25°C in the presence of RNase- and DNase-inhibitors under buffered conditions for 5-10 minutes. After incubation, the samples are analyzed by polyacrylamide gel electrophoresis followed by autoradiography. The presence of a band on the autoradiogram indicates the formation of a complex between DITHP and the radioactive transcript. A band of similar mobility will not be present in samples prepared using control extracts prepared from untransformed cells.

In the alternative, a method to determine the methylase activity of a DITHP measures transfer of radiolabeled methyl groups between a donor substrate and an acceptor substrate. Reaction mixtures (50 µl final volume) contain 15 mM HEPES, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 3% polyvinylalcohol, 1.5 µCi [*methyl*-<sup>3</sup>H]AdoMet (0.375 µM AdoMet) (DuPont-NEN), 0.6 µg DITHP, and acceptor substrate (e.g., 0.4 µg [<sup>35</sup>S]RNA, or 6-mercaptopurine (6-MP) to 1 mM final

concentration). Reaction mixtures are incubated at 30°C for 30 minutes, then 65°C for 5 minutes.

Analysis of [*methyl-<sup>3</sup>H*]RNA is as follows: 1) 50 µl of 2 x loading buffer (20 mM Tris-HCl, pH 7.6, 1 M LiCl, 1 mM EDTA, 1% sodium dodecyl sulphate (SDS)) and 50 µl oligo d(T)-cellulose (10 mg/ml in 1 x loading buffer) are added to the reaction mixture, and incubated at ambient temperature with

- 5 shaking for 30 minutes. 2) Reaction mixtures are transferred to a 96-well filtration plate attached to a vacuum apparatus. 3) Each sample is washed sequentially with three 2.4 ml aliquots of 1 x oligo d(T) loading buffer containing 0.5% SDS, 0.1% SDS, or no SDS. and 4) RNA is eluted with 300 µl of water into a 96-well collection plate, transferred to scintillation vials containing liquid scintillant, and radioactivity determined. Analysis of [*methyl-<sup>3</sup>H*]6-MP is as follows: 1) 500 µl 0.5 M borate buffer, 10 pH 10.0, and then 2.5 ml of 20% (v/v) isoamyl alcohol in toluene are added to the reaction mixtures. 2) The samples mixed by vigorous vortexing for ten seconds. 3) After centrifugation at 700g for 10 minutes, 1.5 ml of the organic phase is transferred to scintillation vials containing 0.5 ml absolute ethanol and liquid scintillant, and radioactivity determined. and 4) Results are corrected for the extraction of 6-MP into the organic phase (approximately 41%).

- 15 An assay for adhesion activity of DITHP measures the disruption of cytoskeletal filament networks upon overexpression of DITHP in cultured cell lines (Reznicek, G.A. et al. (1998) J. Cell Biol. 141:209-225). cDNA encoding DITHP is subcloned into a mammalian expression vector that drives high levels of cDNA expression. This construct is transfected into cultured cells, such as rat kangaroo PtK2 or rat bladder carcinoma 804G cells. Actin filaments and intermediate filaments such 20 as keratin and vimentin are visualized by immunofluorescence microscopy using antibodies and techniques well known in the art. The configuration and abundance of cytoskeletal filaments can be assessed and quantified using confocal imaging techniques. In particular, the bundling and collapse of cytoskeletal filament networks is indicative of DITHP adhesion activity.

- Alternatively, an assay for DITHP activity measures the expression of DITHP on the cell 25 surface. cDNA encoding DITHP is transfected into a non-leukocytic cell line. Cell surface proteins are labeled with biotin (de la Fuente, M.A. et al. (1997) Blood 90:2398-2405). Immunoprecipitations are performed using DITHP-specific antibodies, and immunoprecipitated samples are analyzed using SDS-PAGE and immunoblotting techniques. The ratio of labeled immunoprecipitant to unlabeled immunoprecipitant is proportional to the amount of DITHP expressed on the cell surface.

- 30 Alternatively, an assay for DITHP activity measures the amount of cell aggregation induced by overexpression of DITHP. In this assay, cultured cells such as NIH3T3 are transfected with cDNA encoding DITHP contained within a suitable mammalian expression vector under control of a strong promoter. Cotransfection with cDNA encoding a fluorescent marker protein, such as Green Fluorescent Protein (CLONTECH), is useful for identifying stable transfectants. The amount of cell 35 agglutination, or clumping, associated with transfected cells is compared with that associated with

untransfected cells. The amount of cell agglutination is a direct measure of DITHP activity.

DITHP may recognize and precipitate antigen from serum. This activity can be measured by the quantitative precipitin reaction (Golub, E.S. et al. (1987) Immunology: A Synthesis, Sinauer Associates, Sunderland MA, pages 113-115). DITHP is isotopically labeled using methods known in the art. Various serum concentrations are added to constant amounts of labeled DITHP. DITHP-antigen complexes precipitate out of solution and are collected by centrifugation. The amount of precipitable DITHP-antigen complex is proportional to the amount of radioisotope detected in the precipitate. The amount of precipitable DITHP-antigen complex is plotted against the serum concentration. For various serum concentrations, a characteristic precipitation curve is obtained, in which the amount of precipitable DITHP-antigen complex initially increases proportionately with increasing serum concentration, peaks at the equivalence point, and then decreases proportionately with further increases in serum concentration. Thus, the amount of precipitable DITHP-antigen complex is a measure of DITHP activity which is characterized by sensitivity to both limiting and excess quantities of antigen.

A microtubule motility assay for DITHP measures motor protein activity. In this assay, recombinant DITHP is immobilized onto a glass slide or similar substrate. Taxol-stabilized bovine brain microtubules (commercially available) in a solution containing ATP and cytosolic extract are perfused onto the slide. Movement of microtubules as driven by DITHP motor activity can be visualized and quantified using video-enhanced light microscopy and image analysis techniques. DITHP motor protein activity is directly proportional to the frequency and velocity of microtubule movement.

Alternatively, an assay for DITHP measures the formation of protein filaments in vitro. A solution of DITHP at a concentration greater than the "critical concentration" for polymer assembly is applied to carbon-coated grids. Appropriate nucleation sites may be supplied in the solution. The grids are negative stained with 0.7% (w/v) aqueous uranyl acetate and examined by electron microscopy. The appearance of filaments of approximately 25 nm (microtubules), 8 nm (actin), or 10 nm (intermediate filaments) is a demonstration of protein activity.

DITHP electron transfer activity is demonstrated by oxidation or reduction of NADP. Substrates such as Asn- $\beta$ Gal, biocytidine, or ubiquinone-10 may be used. The reaction mixture contains 1-2 mg/ml HORP, 15 mM substrate, and 2.4 mM NAD(P)<sup>+</sup> in 0.1 M phosphate buffer, pH 7.1 (oxidation reaction), or 2.0 mM NAD(P)H, in 0.1 M Na<sub>2</sub>HPO<sub>4</sub> buffer, pH 7.4 (reduction reaction); in a total volume of 0.1 ml. FAD may be included with NAD, according to methods well known in the art. Changes in absorbance are measured using a recording spectrophotometer. The amount of NAD(P)H is stoichiometrically equivalent to the amount of substrate initially present, and the change in A<sub>340</sub> is a direct measure of the amount of NAD(P)H produced:  $\Delta A_{340} = 6620[\text{NADH}]$ . DITHP activity

is proportional to the amount of NAD(P)H present in the assay. The increase in extinction coefficient of NAD(P)H coenzyme at 340 nm is a measure of oxidation activity, or the decrease in extinction coefficient of NAD(P)H coenzyme at 340 nm is a measure of reduction activity (Dalziel, K. (1963) *J. Biol. Chem.* 238:2850-2858).

5 DITHP transcription factor activity is measured by its ability to stimulate transcription of a reporter gene (Liu, H.Y. et al. (1997) *EMBO J.* 16:5289-5298). The assay entails the use of a well characterized reporter gene construct, LexA<sub>ap</sub>-LacZ, that consists of LexA DNA transcriptional control elements (LexA<sub>ap</sub>) fused to sequences encoding the *E. coli* LacZ enzyme. The methods for constructing and expressing fusion genes, introducing them into cells, and measuring LacZ enzyme activity, are well  
10 known to those skilled in the art. Sequences encoding DITHP are cloned into a plasmid that directs the synthesis of a fusion protein, LexA-DITHP, consisting of DITHP and a DNA binding domain derived from the LexA transcription factor. The resulting plasmid, encoding a LexA-DITHP fusion protein, is introduced into yeast cells along with a plasmid containing the LexA<sub>ap</sub>-LacZ reporter gene. The amount of LacZ enzyme activity associated with LexA-DITHP transfected cells, relative to control cells, is  
15 proportional to the amount of transcription stimulated by the DITHP.

Chromatin activity of DITHP is demonstrated by measuring sensitivity to DNase I (Dawson, B.A. et al. (1989) *J. Biol. Chem.* 264:12830-12837). Samples are treated with DNase I, followed by insertion of a cleavable biotinylated nucleotide analog, 5-[(N-biotinamido)hexanoamido-ethyl-1,3-thiopropionyl-3-aminoallyl]-2'-deoxyuridine 5'-triphosphate using nick-repair techniques well known to  
20 those skilled in the art. Following purification and digestion with EcoRI restriction endonuclease, biotinylated sequences are affinity isolated by sequential binding to streptavidin and biotincellulose.

Another specific assay demonstrates the ion conductance capacity of DITHP using an electrophysiological assay. DITHP is expressed by transforming a mammalian cell line such as COS7, HeLa or CHO with a eukaryotic expression vector encoding DITHP. Eukaryotic expression  
25 vectors are commercially available, and the techniques to introduce them into cells are well known to those skilled in the art. A small amount of a second plasmid, which expresses any one of a number of marker genes such as  $\beta$ -galactosidase, is co-transformed into the cells in order to allow rapid identification of those cells which have taken up and expressed the foreign DNA. The cells are incubated for 48-72 hours after transformation under conditions appropriate for the cell line to allow  
30 expression and accumulation of DITHP and  $\beta$ -galactosidase. Transformed cells expressing  $\beta$ -galactosidase are stained blue when a suitable colorimetric substrate is added to the culture media under conditions that are well known in the art. Stained cells are tested for differences in membrane conductance due to various ions by electrophysiological techniques that are well known in the art. Untransformed cells, and/or cells transformed with either vector sequences alone or  $\beta$ -galactosidase  
35 sequences alone, are used as controls and tested in parallel. The contribution of DITHP to cation or

anion conductance can be shown by incubating the cells using antibodies specific for either DITHP. The respective antibodies will bind to the extracellular side of DITHP, thereby blocking the pore in the ion channel, and the associated conductance.

## 5 XV. Functional Assays

DITHP function is assessed by expressing dithp at physiologically elevated levels in mammalian cell culture systems. cDNA is subcloned into a mammalian expression vector containing a strong promoter that drives high levels of cDNA expression. Vectors of choice include pCMV SPORT (Life Technologies) and pCR3.1 (Invitrogen Corporation, Carlsbad CA), both of which contain the cytomegalovirus promoter. 5-10 µg of recombinant vector are transiently transfected into a human cell line, preferably of endothelial or hematopoietic origin, using either liposome formulations or electroporation. 1-2 µg of an additional plasmid containing sequences encoding a marker protein are co-transfected.

Expression of a marker protein provides a means to distinguish transfected cells from nontransfected cells and is a reliable predictor of cDNA expression from the recombinant vector. Marker proteins of choice include, e.g., Green Fluorescent Protein (GFP; CLONTECH), CD64, or a CD64-GFP fusion protein. Flow cytometry (FCM), an automated laser optics-based technique, is used to identify transfected cells expressing GFP or CD64-GFP and to evaluate the apoptotic state of the cells and other cellular properties.

FCM detects and quantifies the uptake of fluorescent molecules that diagnose events preceding or coincident with cell death. These events include changes in nuclear DNA content as measured by staining of DNA with propidium iodide; changes in cell size and granularity as measured by forward light scatter and 90 degree side light scatter; down-regulation of DNA synthesis as measured by decrease in bromodeoxyuridine uptake; alterations in expression of cell surface and intracellular proteins as measured by reactivity with specific antibodies; and alterations in plasma membrane composition as measured by the binding of fluorescein-conjugated Annexin V protein to the cell surface. Methods in flow cytometry are discussed in Ormerod, M. G. (1994) Flow Cytometry, Oxford, New York NY.

The influence of DITHP on gene expression can be assessed using highly purified populations of cells transfected with sequences encoding DITHP and either CD64 or CD64-GFP. CD64 and CD64-GFP are expressed on the surface of transfected cells and bind to conserved regions of human immunoglobulin G (IgG). Transfected cells are efficiently separated from nontransfected cells using magnetic beads coated with either human IgG or antibody against CD64 (DYNAL, Inc., Lake Success NY). mRNA can be purified from the cells using methods well known by those of skill in the art.

Expression of mRNA encoding DITHP and other genes of interest can be analyzed by northern analysis

or microarray techniques.

#### XVI. Production of Antibodies

DITHP substantially purified using polyacrylamide gel electrophoresis (PAGE; see, e.g.,  
 5 Harrington, M.G. (1990) *Methods Enzymol.* 182:488-495), or other purification techniques, is used to immunize rabbits and to produce antibodies using standard protocols.

Alternatively, the DITHP amino acid sequence is analyzed using LASERGENE software (DNASTAR) to determine regions of high immunogenicity, and a corresponding peptide is synthesized and used to raise antibodies by means known to those of skill in the art. Methods for selection of  
 10 appropriate epitopes, such as those near the C-terminus or in hydrophilic regions are well described in the art. (See, e.g., Ausubel, 1995, *supra*, Chapter 11.)

Typically, peptides 15 residues in length are synthesized using an ABI 431A peptide synthesizer (PE Biosystems) using fmoc-chemistry and coupled to KLH (Sigma) by reaction with N-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) to increase immunogenicity. (See, e.g.,  
 15 Ausubel, *supra*.) Rabbits are immunized with the peptide-KLH complex in complete Freund's adjuvant. Resulting antisera are tested for antipeptide activity by, for example, binding the peptide to plastic, blocking with 1% BSA, reacting with rabbit antisera, washing, and reacting with radioiodinated goat anti-rabbit IgG. Antisera with antipeptide activity are tested for anti-DITHP activity using protocols well known in the art, including ELISA, RIA, and immunoblotting.

20

#### XVII. Purification of Naturally Occurring DITHP Using Specific Antibodies

Naturally occurring or recombinant DITHP is substantially purified by immunoaffinity chromatography using antibodies specific for DITHP. An immunoaffinity column is constructed by covalently coupling anti-DITHP antibody to an activated chromatographic resin, such as  
 25 CNBr-activated SEPHAROSE (Amersham Pharmacia Biotech). After the coupling, the resin is blocked and washed according to the manufacturer's instructions.

Media containing DITHP are passed over the immunoaffinity column, and the column is washed under conditions that allow the preferential absorbance of DITHP (e.g., high ionic strength buffers in the presence of detergent). The column is eluted under conditions that disrupt  
 30 antibody/DITHP binding (e.g., a buffer of pH 2 to pH 3, or a high concentration of a chaotrope, such as urea or thiocyanate ion), and DITHP is collected.

#### XVIII. Identification of Molecules Which Interact with DITHP

DITHP, or biologically active fragments thereof, are labeled with <sup>125</sup>I Bolton-Hunter reagent.  
 35 (See, e.g., Bolton, A.E. and W.M. Hunter (1973) *Biochem. J.* 133:529-539.) Candidate molecules

previously arrayed in the wells of a multi-well plate are incubated with the labeled DITHP, washed, and any wells with labeled DITHP complex are assayed. Data obtained using different concentrations of DITHP are used to calculate values for the number, affinity, and association of DITHP with the candidate molecules.

- 5           Alternatively, molecules interacting with DITHP are analyzed using the yeast two-hybrid system as described in Fields, S. and O. Song (1989) Nature 340:245-246, or using commercially available kits based on the two-hybrid system, such as the MATCHMAKER system (CLONTECH).

- DITHP may also be used in the PATHCALLING process (CuraGen Corp., New Haven CT) which employs the yeast two-hybrid system in a high-throughput manner to determine all interactions  
10   between the proteins encoded by two large libraries of genes (Nandabalan, K. et al. (2000) U.S. Patent No. 6,057,101).

- All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention  
15   will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the above-described modes for carrying out the invention which are obvious to those skilled in the field of molecular biology or related fields are intended to be within the  
20   scope of the following claims.



TABLE 1

SEQ ID NO:	Template ID	GI Number	Probability Score	Annotation
1	061149.1.i	g3073775	3.20E-31	heparan-sulfate O-sulfotransferase
2	404508.3.j	g55535	1.30E-35	100 kDa protein
3	441227.2.j	g2896147	2.80E-49	Human alpha-methylacyl-CoA racemase mRNA, complete cds.
4	277927.2	g2708639	0	carbonic anhydrase precursor
5	475311.1	g6815284	0	Homo sapiens methionine adenosyltransferase regulatory beta subunit mRNA, complete cds.
6	13039.2	g35790	1.70E-12	protein-tyrosine phosphatase
7	238005.4	g4321619	9.40E-96	seven transmembrane domain orphan receptor
8	345322.1.j	g206352	1.50E-15	protein phosphatase inhibitor-1 protein
9	348094.6.j	g3876117	3.80E-77	similarity to mouse nek1 protein kinase
10	233678.1	g6179911	0	Homo sapiens ADP-ribosylation factor binding protein GGA1 (GGA1) mRNA, complete cds.
11	312243.1	g3925265	6.90E-21	similar to Probable rabGAP domains
12	425487.3	g6179913	1.00E-154	Homo sapiens ADP-ribosylation factor binding protein GGA2 (GGA2) mRNA, complete cds.
13	345813.3.j	g2088550	0	Human hereditary haemochromatosis region, histone 2A-like protein gene, phosphate transporter (HLA-H) gene, Rarrel gene, and sodium
14	005861.1.j	g1263080	4.00E-52	phosphatase transporter (NP13) gene, complete cds.
15	028008.3.j	g2463648	5.00E-37	Human mariner1 transposase gene, complete consensus sequence.
16	346078.5.j	g3746839	0	snRNP core Sm protein homolog Sm-X5
17	394637.1.j	g1263080	0	Human 45kDa splicing factor mRNA, complete cds.
18	222429.3	g337451	1.90E-17	Human mariner1 transposase gene, complete consensus sequence.
19	366739.2	g5524926	0	hnRNP type A/B protein
20	474635.6	g3880433	4.90E-16	Homo sapiens mRNA for deoxyribonuclease III (dm3 gene), similar to mitochondrial RNA splicing MSTR4 like protein; cDNA EST EMBL:CO9217 comes from this gene
21	228470.1.j	g514373	1.00E-80	Opticoid-binding cell adhesion molecule
22	407090.5.j	g500858	1.40E-10	50 kDa lectin
23	068194.1.j	g397947	1.20E-23	thioredoxin
24	411449.2	g5295993	0	Homo sapiens SDHD gene for small subunit of cytochrome b of succinate dehydrogenase, complete cds.

TABLE 1

SEQ ID NO:	Template ID	GI Number	Probability Score	Annotation
25	18549.2	g4455608	1.50E-20	dJ742C19.2 (APOBEC1 (Apolipoprotein B mRNA editing protein) and Phorbol 1 LIKE)
26	236043.3	g562263	1.20E-94	topomodulin
27	445433.2	g28251	6.00E-25	Human mRNA for beta-actin.
28	344630.7j	g40828	5.40E-30	Hyd gamma (homologous to the periplasmic hydrogenase)
29	257121.2	g550123	2.30E-163	GED-154
30	243794.1j	g36131	0	Human mRNA for ribosomal protein L32.
31	442085.1j	g437878	4.50E-25	ribosomal protein S24
32	377061.3j	g1016712	1.40E-28	Fos-related antigen
33	427939.17j	g488555	1.20E-85	zinc finger protein ZNF135
34	430569.2j	g487784	9.00E-16	Human zinc finger protein ZNF136.
35	444689.1j	g487784	1.00E-26	Human zinc finger protein ZNF136.
36	445198.1j	g498152	3.70E-35	hcd0946 protein is Kruppel-related
37	84399.1	g207696	2.80E-17	zinc finger protein
38	350044.1	g1613847	5.00E-10	Human zinc finger protein zfp6 (ZF6) mRNA, partial cds.
39	441329.2	g3005231	4.10E-78	Zn-finger-like protein; similar to Z98745 (P1Dg2924250)
40	442401.2	g498722	4.00E-62	Human HZF2 mRNA for zinc finger protein.
41	444933.2	g456269	1.80E-20	zinc finger protein 30
42	481129.4	g3876716	3.10E-15	similar to Zinc finger, C3HC4 type (RING finger)
43	481999.1	g4519269	3.00E-45	Human ZK1 mRNA for Kruppel-type zinc finger protein, complete cds.
44	233814.1j	g1326367	7.50E-11	Similar to the mitochondrial carrier family
45	351376.4j	g517226	7.40E-15	mitochondrial ATPase inhibitor
46	338992.1	g414797	2.80E-253	pyruvate dehydrogenase phosphatase
47	200587.3j	g722379	7.50E-23	similar to NIFS protein (nitrogen fixation)
48	246727.5j	g337456	4.00E-25	Human ribonucleoprotein (Lo) mRNA, 3' end
49	407087.3j	g1176422	7.40E-72	rhophilin
50	441779.1j	g1304381	8.10E-56	hemoglobin alpha chain
51	206603.1	g4101720	4.00E-14	lymphocyte specific formin related protein
52	435694.2	g4572570	3.00E-22	Homo sapiens lun dimerization protein gene, partial cds; c'os gene, complete cds; and unknown gene.

TABLE 2

SEQ ID NO:	Template ID	Start	Stop	Frame	Plam Hit	Plam Description	E-value
6	13039.2	568	801	forward 1	In3	Fibrinectin type III domain	3.00E-09
9	348094.6j	255	779	forward 3	pkinase	Eukaryotic protein kinase domain	1.10E-53
9	348094.6j	782	967	forward 2	pkinase	Eukaryotic protein kinase domain	2.70E-08
10	233678.1	149	514	forward 2	VHS	VHS domain	1.30E-06
11	312243.1	703	1263	forward 1	TBC	TBC domain	2.20E-07
12	425487.3	78	407	forward 3	VHS	VHS domain	2.90E-09
16	346078.5j	844	978	forward 1	G-patch	G-patch domain	8.00E-08
20	474635.6	258	530	forward 3	mito_carr	Mitochondrial carrier proteins	4.10E-17
20	474635.6	2449	2652	forward 1	mito_carr	Mitochondrial carrier proteins	2.40E-10
21	228470.1j	312	515	forward 3	Ig	Immunoglobulin domain	6.20E-08
29	257121.2	344	541	forward 2	IBR	IBR domain	7.70E-23
30	243794.1j	456	740	forward 3	Ribosomal_L32e	Ribosomal protein L32	8.00E-20
30	243794.1j	231	326	forward 3	Ribosomal_S14	Ribosomal protein S14p/S29e	1.40E-12
31	442085.1j	149	400	forward 2	Ribosomal_S24e	Ribosomal protein S24e	4.70E-40
33	427939.17j	1002	1070	forward 3	zf-C2H2	Zinc finger, C2H2 type	1.70E-07
33	427939.17j	589	657	forward 1	zf-C2H2	Zinc finger, C2H2 type	4.80E-07
35	444689.1j	170	274	forward 2	KRAB	KRAB box	8.70E-06
36	445198.1j	80	268	forward 2	KRAB	KRAB box	2.90E-42
38	350044.1	76	240	forward 1	KRAB	KRAB box	4.40E-12
38	350044.1	895	963	forward 1	zf-C2H2	Zinc finger, C2H2 type	8.40E-06
39	441329.2	684	965	forward 3	SCAN	SCAN domain	5.30E-48
40	442401.2	206	394	forward 2	KRAB	KRAB box	2.40E-41
41	444933.2	292	480	forward 1	KRAB	KRAB box	1.40E-41
43	481999.1	371	547	forward 2	KRAB	KRAB box	1.90E-15
43	481999.1	839	907	forward 2	zf-C2H2	Zinc finger, C2H2 type	1.40E-04
44	233814.1j	356	625	forward 2	mito_carr	Mitochondrial carrier proteins	2.10E-19
46	338992.1	1156	1680	forward 1	PP2C	Protein phosphatase 2C	1.90E-54
46	338992.1	840	1184	forward 3	PP2C	Protein phosphatase 2C	3.30E-10
47	200587.3j	146	1276	forward 2	aminotran_5	Aminotransferases class-V	3.20E-32
50	441779.1j	76	498	forward 1	globin	Globin	4.20E-53

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TABLE 3

SEQ ID NO:	Template ID	Start	Stop	Frame	Domain Type
2	404508.3.j	468	548	reverse 3	TM
2	404508.3.j	549	626	forward 3	SP
2	404508.3.j	348	428	reverse 3	TM
2	404508.3.j	430	513	reverse 1	SP
2	404508.3.j	459	539	forward 3	TM
3	441227.2.j	523	606	forward 1	SP
6	13039.2	1507	1581	forward 1	TM
7	238005.4	887	973	forward 2	SP
7	238005.4	1691	1783	forward 2	SP
7	238005.4	1583	1678	forward 2	SP
7	238005.4	566	643	forward 2	TM
9	348094.6.j	1994	2077	forward 2	TM
9	348094.6.j	1871	1948	reverse 2	TM
9	348094.6.j	2046	2117	forward 3	TM
9	348094.6.j	2112	2195	reverse 3	TM
9	348094.6.j	2035	2115	forward 1	TM
9	348094.6.j	2920	3000	forward 1	TM
10	233678.1	2412	2495	forward 3	SP
11	312243.1	528	614	forward 3	SP
16	346078.5.j	1406	1492	forward 2	TM
17	394637.1.j	36	122	forward 3	TM
18	222429.3	646	726	forward 1	SP
19	366739.2	791	880	forward 2	SP
19	366739.2	1115	1207	forward 2	SP
20	474635.6	1293	1370	forward 3	TM
22	407090.5.j	2955	3038	reverse 3	SP
24	411449.2	283	366	forward 1	SP
27	445433.2	123	203	forward 3	TM
29	257121.2	3656	3736	forward 2	TM
29	257121.2	851	934	forward 2	TM
46	338992.1	1601	1690	forward 2	SP
46	338992.1	1478	1561	forward 2	SP

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TABLE 4

SEQ ID NO:	Template ID	Component ID	Start	Stop
4	277927.2	2706884H1	1	199
4	277927.2	4317170F6	1	384
4	277927.2	2706884F6	1	320
4	277927.2	g1062337	36	388
4	277927.2	g1064195	39	230
4	277927.2	2862653H1	42	117
4	277927.2	494376H1	53	305
4	277927.2	494376R7	53	383
4	277927.2	494376R6	53	492
4	277927.2	g1275473	57	586
4	277927.2	g2055898	59	543
4	277927.2	2707487H1	64	162
4	277927.2	491071H1	74	319
4	277927.2	g1975146	80	403
4	277927.2	g1142488	84	471
4	277927.2	g1839772	84	214
4	277927.2	g1012520	84	292
4	277927.2	3396290H1	86	344
4	277927.2	5896091H1	246	523
4	277927.2	g946240	246	551
4	277927.2	g943428	247	449
5	475311.1	1948746H1	1	233
5	475311.1	g614088	12	271
5	475311.1	5644994H1	32	287
5	475311.1	g645077	37	260
5	475311.1	g668814	40	366
5	475311.1	g645713	42	263
5	475311.1	g645045	42	359
5	475311.1	4297187H1	46	315
5	475311.1	3486028H1	53	385
5	475311.1	g683448	54	438
5	475311.1	g645139	54	252
5	475311.1	g645138	54	316
5	475311.1	g670408	54	433
5	475311.1	g646240	56	444
5	475311.1	g562499	56	444
5	475311.1	g4113162	56	511
5	475311.1	g2003099	59	473
5	475311.1	g815985	71	481
5	475311.1	g674342	77	421
5	475311.1	4151149H1	88	355
5	475311.1	3843963H1	88	393
5	475311.1	5029373H1	92	346
5	475311.1	3584334H1	98	435
5	475311.1	g3015753	105	441
5	475311.1	g831378	105	457
5	475311.1	g900504	105	401
5	475311.1	g817737	105	402
5	475311.1	g817003	105	418
5	475311.1	g812268	105	397

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SEQ ID NO:	Template ID	Component ID	Start	Stop
5	475311.1	g2052991	105	462
5	475311.1	g3922575	105	509
5	475311.1	g2884797	105	548
5	475311.1	g2354824	105	375
5	475311.1	g2788503	105	214
5	475311.1	g797560	105	342
5	475311.1	g820611	105	346
5	475311.1	g821223	105	347
5	475311.1	g1678742	138	543
5	475311.1	5590296H1	150	396
5	475311.1	2446939H1	155	407
5	475311.1	4178404H1	164	434
5	475311.1	4970343H1	179	455
5	475311.1	g1720508	218	502
5	475311.1	4816085H1	242	326
5	475311.1	g1296172	248	789
5	475311.1	5778021H1	256	544
5	475311.1	1579546F6	263	712
5	475311.1	1227469H1	263	535
5	475311.1	1951396H1	287	527
5	475311.1	g1980791	307	631
5	475311.1	3369048H1	322	455
5	475311.1	261669H1	325	559
5	475311.1	1005556H1	344	651
5	475311.1	g1987083	364	686
5	475311.1	3153327H1	364	683
5	475311.1	5086472H1	372	552
5	475311.1	g2038507	365	782
5	475311.1	3791124H1	366	623
5	475311.1	3342191H1	383	665
5	475311.1	2727001H1	403	658
5	475311.1	5691335H1	421	697
5	475311.1	2246168H1	434	702
5	475311.1	4186012H1	582	933
5	475311.1	2630947H1	653	899
5	475311.1	g1444007	793	1005
6	13039.2	4189083H1	3573	3883
6	13039.2	g3802900	3582	3888
6	13039.2	2881380H1	3588	3897
6	13039.2	g864431	3590	3852
6	13039.2	g1481926	3594	3881
6	13039.2	g667472	3600	3883
6	13039.2	g3154327	3607	3891
6	13039.2	4583956H1	3611	3880
6	13039.2	g776778	3615	3887
6	13039.2	2381092H1	3622	3854
6	13039.2	3810284H1	3623	3895
6	13039.2	4906362H2	3624	3904
6	13039.2	2202368H1	3632	3875
6	13039.2	g4072852	3636	4070

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TABLE 4

SEQ ID NO:	Template ID	Component ID	Start	Stop
6	13039.2	2561132H1	3646	3748
6	13039.2	1294323F1	3648	3884
6	13039.2	1294323H1	3648	3780
6	13039.2	446234H1	3649	3871
6	13039.2	4774923H1	3650	3935
6	13039.2	g789650	3651	3882
6	13039.2	g1220073	3652	3888
6	13039.2	g776622	3658	3886
6	13039.2	g1522080	3663	3883
6	13039.2	449477H1	3667	3875
6	13039.2	2293709H1	3669	3884
6	13039.2	g1114992	3695	3885
6	13039.2	g667182	3700	3883
6	13039.2	g1162269	3703	4063
6	13039.2	g1773892	3703	4081
6	13039.2	g3887238	3705	4083
6	13039.2	3542520H1	3707	3870
6	13039.2	4633040H1	3727	4003
6	13039.2	g1128515	3763	4081
6	13039.2	2804481H1	3793	3899
6	13039.2	556467R6	3816	3871
6	13039.2	556467H1	3816	3871
6	13039.2	g683155	3819	4075
6	13039.2	g878104	3824	4075
6	13039.2	2945643H1	3841	4076
6	13039.2	g821501	3869	4087
6	13039.2	g782508	3869	4083
6	13039.2	2590326H2	4016	4075
6	13039.2	4535971T6	1081	1596
6	13039.2	2887511H1	1137	1386
6	13039.2	5060591H1	1078	1278
6	13039.2	2261086H1	1154	1385
6	13039.2	3535673H1	1214	1493
6	13039.2	1951291H1	1264	1453
6	13039.2	488798H1	1330	1587
6	13039.2	2634402H1	1381	1594
6	13039.2	2135368H1	1421	1684
6	13039.2	2135368F6	1421	1863
6	13039.2	2837860T6	3920	4029
6	13039.2	2837860F6	3927	4069
6	13039.2	2837860H1	3927	4069
6	13039.2	3536266H1	3936	4027
6	13039.2	3536268H1	3937	4038
6	13039.2	g776777	3055	3279
6	13039.2	g1856268	3100	3505
6	13039.2	2604507H1	3110	3384
6	13039.2	5065865H1	3071	3344
6	13039.2	3472443H1	3077	3330
6	13039.2	5017370H1	3114	3384
6	13039.2	2767212H1	3090	3334

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TABLE 4

SEQ ID NO:	Template ID	Component ID	Start	Stop
6	13039.2	790129R1	3121	3597
6	13039.2	2690375H1	3094	3357
6	13039.2	2411049H1	3099	3334
6	13039.2	g1976982	1594	1956
6	13039.2	2642416H1	1599	1728
6	13039.2	g1476955	1606	2005
6	13039.2	g1062967	1606	1901
6	13039.2	g1481925	1606	1784
6	13039.2	3136852H1	1738	2037
6	13039.2	2500241H1	1778	2029
6	13039.2	2788782H1	1863	2121
6	13039.2	874642H1	1862	2024
6	13039.2	g1200951	1869	2062
6	13039.2	4535987H1	1879	2008
6	13039.2	4536041H1	1884	2151
6	13039.2	1921318T6	3558	4028
6	13039.2	g2458968	3562	3880
6	13039.2	3434402H1	1	237
6	13039.2	g1774870	25	371
6	13039.2	5090377H1	28	270
6	13039.2	5090377F6	28	341
6	13039.2	g2243533	71	456
6	13039.2	4535971H1	377	641
6	13039.2	4535971F6	377	900
6	13039.2	2738090H1	634	871
6	13039.2	2738090F6	634	1152
6	13039.2	4032617H1	652	788
6	13039.2	3247382H1	687	986
6	13039.2	2886124H1	2974	3263
6	13039.2	2886134H1	2974	3255
6	13039.2	2882975H1	2974	3267
6	13039.2	731806R1	2984	3499
6	13039.2	3715202H1	2984	3092
6	13039.2	g1993687	2985	3312
6	13039.2	731806H1	2984	3258
6	13039.2	2468144H1	2990	3229
6	13039.2	1907815H1	2991	3218
6	13039.2	4148570H1	3006	3269
6	13039.2	g778804	3015	3330
6	13039.2	3256396H1	3017	3269
6	13039.2	g789649	3021	3255
6	13039.2	4983180H1	3028	3311
6	13039.2	g865926	3030	3351
6	13039.2	4983190H1	3028	3302
6	13039.2	998221R1	3039	3594
6	13039.2	998221H1	3039	3328
6	13039.2	4650828H1	3041	3328
6	13039.2	g776667	3055	3330
6	13039.2	3699551H1	759	1043
6	13039.2	2784137H1	768	999



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SEQ ID NO:	Template ID	Component ID	Start	Stop
6	13039.2	5092174H1	863	1136
6	13039.2	2736123H1	901	1143
6	13039.2	2736123F6	901	1374
6	13039.2	2841123H2	964	1052
6	13039.2	g1801914	984	1330
6	13039.2	3627415H1	990	1242
6	13039.2	2049675H1	1064	1318
6	13039.2	2783785F6	1067	1509
6	13039.2	2783785H1	1067	1319
6	13039.2	4764557H1	2700	2895
6	13039.2	g862228	2711	2935
6	13039.2	4977405H1	2718	2987
6	13039.2	4324144H1	2725	3007
6	13039.2	5195156H1	2730	2831
6	13039.2	4625438H1	2738	2992
6	13039.2	2881531H1	2740	3023
6	13039.2	1545834H1	2740	2953
6	13039.2	3214807H1	2740	2990
6	13039.2	1312356H1	2750	2954
6	13039.2	1312307H1	2750	2979
6	13039.2	g1238200	3569	3881
6	13039.2	g792128	3570	3881
6	13039.2	g3109745	3571	3878
6	13039.2	g789837	2241	2520
6	13039.2	g666844	2248	2506
6	13039.2	g793332	2260	2481
6	13039.2	g862227	2260	2522
6	13039.2	3321110H1	2293	2570
6	13039.2	g1636044	2306	2530
6	13039.2	3738558H1	2316	2611
6	13039.2	5700029H1	2317	2575
6	13039.2	5030623H1	2361	2636
6	13039.2	3321569H2	2364	2614
6	13039.2	3484339H1	2365	2701
6	13039.2	g317338	2375	2655
6	13039.2	4922854H1	2377	2683
6	13039.2	1382370H1	2377	2615
6	13039.2	3364673H1	2393	2653
6	13039.2	4014451H1	2393	2645
6	13039.2	4296168H1	2429	2676
6	13039.2	4296144H1	2429	2683
6	13039.2	4295505H1	2429	2696
6	13039.2	4775793H1	2466	2741
6	13039.2	5190065H1	1477	1620
6	13039.2	g2013579	1518	1807
6	13039.2	3201887H1	1570	1835
6	13039.2	g708914	1436	1737
6	13039.2	g691657	1479	1801
6	13039.2	489678H1	1579	1818
6	13039.2	044642H1	2172	2328

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TABLE 4

SEQ ID NO:	Template ID	Component ID	Start	Stop
6	13039.2	3129828H1	2163	2472
6	13039.2	5702193H1	2183	2423
6	13039.2	g866587	2185	2551
6	13039.2	4968731H1	2217	2474
6	13039.2	4969450H1	2217	2498
6	13039.2	g434208	2221	2510
6	13039.2	g666222	3533	3878
6	13039.2	1861085T6	3554	3841
6	13039.2	g1476956	3554	3883
6	13039.2	2472134H1	1917	2158
6	13039.2	2460193H1	1973	2223
6	13039.2	5544753H1	1977	2125
6	13039.2	1894461H1	1994	2224
6	13039.2	4989013H1	2004	2267
6	13039.2	2504842H1	2018	2250
6	13039.2	3817686H1	2039	2289
6	13039.2	1709583F6	2086	2637
6	13039.2	1709583H1	2086	2292
6	13039.2	5496588H1	2117	2221
6	13039.2	g618254	2133	2456
6	13039.2	2302357H2	2134	2358
6	13039.2	3076667H1	2142	2408
6	13039.2	g4096030	3440	3883
6	13039.2	g4394182	3444	3889
6	13039.2	g3298631	3450	3883
6	13039.2	g4152977	3452	3888
6	13039.2	4150667H1	3456	3752
6	13039.2	5036313H1	3454	3744
6	13039.2	g4110089	3455	3887
6	13039.2	g3110528	3467	3881
6	13039.2	g3884368	3468	3883
6	13039.2	g2631703	3472	3882
6	13039.2	g4329108	3483	3871
6	13039.2	g4307404	3484	3891
6	13039.2	g3887724	3487	3890
6	13039.2	5679544H1	3488	3784
6	13039.2	4643394H1	3488	3741
6	13039.2	231550R1	3496	3891
6	13039.2	231550H1	3496	3719
6	13039.2	g2659034	3504	3678
6	13039.2	3635404H1	3504	3814
6	13039.2	2736123T6	3515	3839
6	13039.2	231550F1	3523	3882
6	13039.2	g3596402	3524	3881
6	13039.2	2736217H1	3526	3796
6	13039.2	2368391H2	3323	3556
6	13039.2	4761918H1	3324	3582
6	13039.2	3162730H1	3331	3637
6	13039.2	2911248H1	3338	3625
6	13039.2	780353H1	3345	3665

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SEQ ID NO:	Template ID	Component ID	Start	Stop
6	13039.2	1233680H1	3349	3681
6	13039.2	1233680F1	3349	3971
6	13039.2	3792837H1	3351	3635
6	13039.2	g2357493	3351	3602
6	13039.2	2005695H1	3354	3432
6	13039.2	g3087116	3355	3891
6	13039.2	3038679H1	3359	3631
6	13039.2	731806F1	3361	3882
6	13039.2	g3070256	3362	3878
6	13039.2	g3049253	3364	3885
6	13039.2	g3049251	3366	3885
6	13039.2	g3070255	3369	3878
6	13039.2	g3076969	3370	3878
6	13039.2	g4292218	3374	3875
6	13039.2	2379289H1	3376	3605
6	13039.2	6026190H1	3375	3685
6	13039.2	2887871H1	3378	3685
6	13039.2	g3841328	3384	3878
6	13039.2	g4073143	3385	3881
6	13039.2	602339H1	3386	3668
6	13039.2	g2901329	3390	3885
6	13039.2	g4153184	3401	3882
6	13039.2	1862447T6	3417	3836
6	13039.2	2738090T6	3429	3840
6	13039.2	1709583T6	3431	3837
6	13039.2	2870017H1	3229	3528
6	13039.2	2859049H1	3229	3512
6	13039.2	1822377H1	3237	3499
6	13039.2	3601353H1	3245	3577
6	13039.2	2760117H1	3247	3543
6	13039.2	2751935H1	3247	3520
6	13039.2	1921318H1	3255	3535
6	13039.2	1921318R6	3255	3696
6	13039.2	4797434H1	3262	3539
6	13039.2	g778894	3266	3611
6	13039.2	g855990	3266	3591
6	13039.2	2135368T6	3266	3840
6	13039.2	3975369H1	3275	3387
6	13039.2	3442784H1	3283	3547
6	13039.2	1832656H1	3299	3596
6	13039.2	2219316H1	3317	3573
6	13039.2	4761911H1	3323	3598
6	13039.2	g1238488	3188	3379
6	13039.2	g877900	3195	3513
6	13039.2	g2148718	3196	3847
6	13039.2	1736290H1	3199	3439
6	13039.2	1734801H1	3199	3448
6	13039.2	1734817H1	3199	3447
6	13039.2	3812780H1	3201	3506
6	13039.2	3814117H1	3202	3367

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SEQ ID NO:	Template ID	Component ID	Start	Stop
6	13039.2	2404648H1	3213	3450
6	13039.2	1736463H1	3215	3509
6	13039.2	5019558H1	3217	3470
6	13039.2	5559285H1	3217	3498
6	13039.2	793056H1	3123	3357
6	13039.2	790129H1	3134	3376
6	13039.2	2671569H1	3169	3272
6	13039.2	4255130H1	3176	3267
6	13039.2	4164759H1	3170	3434
6	13039.2	4255178H1	3175	3459
6	13039.2	1861085F6	3562	3882
6	13039.2	g1203064	3563	3884
6	13039.2	1861177H1	3563	3888
6	13039.2	2803220H1	2525	2792
6	13039.2	4879807H1	2476	2740
6	13039.2	1627449H1	2527	2661
6	13039.2	2660611H1	2481	2734
6	13039.2	3449673H1	2502	2757
6	13039.2	5039738H2	2535	2749
6	13039.2	4595615H1	2538	2813
6	13039.2	1862447F6	2566	3056
6	13039.2	1862447H1	2566	2831
6	13039.2	4251362H1	2587	2852
6	13039.2	g864534	2601	2918
6	13039.2	g1949105	2608	2883
6	13039.2	4831252H1	2612	2740
6	13039.2	4830165H1	2612	2824
6	13039.2	g1522198	2629	2917
6	13039.2	2962002H1	2642	2938
6	13039.2	4111111H1	2668	2941
6	13039.2	4581818H1	2674	2970
6	13039.2	3480168H1	2674	2966
6	13039.2	059851H1	2688	2875
6	13039.2	3463785H1	2827	3087
6	13039.2	1379162H1	2833	3074
6	13039.2	827278H1	2833	3141
6	13039.2	1379162F1	2833	3380
6	13039.2	827278R1	2833	3428
6	13039.2	4970664H1	2852	3138
6	13039.2	3765903H1	2856	3173
6	13039.2	g828803	2876	3107
6	13039.2	2912660H1	2876	3139
6	13039.2	2945011H1	2929	3231
6	13039.2	1900343H1	2930	3184
6	13039.2	4177622H1	2939	3215
6	13039.2	2714979H1	2941	3205
6	13039.2	5098921H1	2946	3241
6	13039.2	4589878H1	2963	3194
6	13039.2	1350496H1	2966	3227
6	13039.2	1350496F1	2966	3639

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SEQ ID NO:	Template ID	Component ID	Start	Stop
6	13039.2	g793050	2751	2936
6	13039.2	2396484H1	2751	2985
6	13039.2	3677377H1	2764	2931
6	13039.2	1613610H1	2765	2976
6	13039.2	3677367H1	2765	3072
6	13039.2	3813522H1	2772	3037
6	13039.2	4688970H1	2782	3032
6	13039.2	4889047H1	2811	3096
6	13039.2	4550845H1	2811	2992
6	13039.2	g1986776	2811	3068
6	13039.2	2943108H1	2816	3136
6	13039.2	919863H1	2816	3094
6	13039.2	2220586H1	2818	3075
7	238005.4	3494842H1	1	146
7	238005.4	1513955H1	19	211
7	238005.4	1513931H1	19	189
7	238005.4	1895354H1	24	255
7	238005.4	3216547H1	53	287
7	238005.4	2492948H1	210	525
7	238005.4	3393775H1	226	487
7	238005.4	3110735H1	237	520
7	238005.4	2114053H1	238	500
7	238005.4	2639154H1	229	480
7	238005.4	2639154F6	229	615
7	238005.4	3489530H1	251	540
7	238005.4	2452873H1	253	499
7	238005.4	2452873F6	253	419
7	238005.4	3149681H1	258	499
7	238005.4	2952379H1	265	542
7	238005.4	2466034H1	291	507
7	238005.4	3074678H1	330	611
7	238005.4	g2003143	378	830
7	238005.4	2310518R6	454	935
7	238005.4	2310518H1	454	735
7	238005.4	2466818H1	500	732
7	238005.4	3243701H1	503	764
7	238005.4	3108380H1	506	773
7	238005.4	4250192H1	506	710
7	238005.4	3688838H1	527	810
7	238005.4	2312916H1	543	816
7	238005.4	4087821H1	570	878
7	238005.4	g3173776	593	909
7	238005.4	3960672H2	599	874
7	238005.4	1830753H1	644	897
7	238005.4	3936919H1	690	871
7	238005.4	3183381H1	691	942
7	238005.4	3165765H1	714	1037
7	238005.4	3231218H1	762	1012
7	238005.4	5273755H1	763	1032
7	238005.4	4710712H1	876	997

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SEQ ID NO:	Template ID	Component ID	Start	Stop
7	238005.4	g847384	989	1313
7	238005.4	5810260H1	999	1303
7	238005.4	2046234F6	1017	1417
7	238005.4	4547319H1	1024	1320
7	238005.4	5302724H1	1041	1284
7	238005.4	5267284H1	1053	1283
7	238005.4	4862949H1	1072	1245
7	238005.4	2893053H1	1078	1355
7	238005.4	2893053F6	1078	1449
7	238005.4	3408366H1	1120	1378
7	238005.4	5427753H1	1132	1350
7	238005.4	2046234H1	1135	1417
7	238005.4	g846587	1153	1408
7	238005.4	1978835H1	1173	1431
7	238005.4	5187046H1	1212	1303
7	238005.4	5049433F6	1212	1740
7	238005.4	5069619H1	1213	1457
7	238005.4	079996H1	1221	1447
7	238005.4	2755107H1	1244	1546
7	238005.4	2994580H1	1250	1546
7	238005.4	1357968H1	1253	1465
7	238005.4	2486058H1	1290	1528
7	238005.4	3155023H1	1308	1607
7	238005.4	1405122H1	1358	1634
7	238005.4	074212H1	1360	1547
7	238005.4	g1775652	1362	1565
7	238005.4	5426560H1	1368	1643
7	238005.4	2452873T6	1376	1902
7	238005.4	5597619H1	1385	1634
7	238005.4	2640268T6	1394	1890
7	238005.4	2971688H1	1399	1715
7	238005.4	2310518T6	1425	1902
7	238005.4	522334H1	1421	1673
7	238005.4	4787913H1	1436	1716
7	238005.4	6093903H1	1439	1775
7	238005.4	3716621H1	1449	1751
7	238005.4	3435261H1	1451	1718
7	238005.4	3256708H1	1454	1740
7	238005.4	769094H1	1454	1685
7	238005.4	g2526289	1458	1946
7	238005.4	4974673H1	1461	1728
7	238005.4	3870931H1	1471	1787
7	238005.4	4701192H1	1471	1746
7	238005.4	g4113880	1481	1936
7	238005.4	5703513H1	1508	1795
7	238005.4	5867713H1	1510	1740
7	238005.4	g3430405	1520	1936
7	238005.4	g4304488	1527	1925
7	238005.4	g3446639	1532	1952
7	238005.4	g3843850	1534	1956

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SEQ ID NO:	Template ID	Component ID	Start	Stop
7	238005.4	5049433T6	1538	1924
7	238005.4	g3895204	1538	1945
7	238005.4	3343709T6	1541	1905
7	238005.4	g4267940	1548	1946
7	238005.4	2269156H1	1556	1832
7	238005.4	3211050H1	1556	1795
7	238005.4	3858314H1	1561	1865
7	238005.4	3858359H1	1561	1845
7	238005.4	3861114H1	1561	1868
7	238005.4	4215258H1	1567	1843
7	238005.4	g1647982	1573	1944
7	238005.4	6106957H1	1583	1903
7	238005.4	4974030H1	1586	1853
7	238005.4	g518289	1588	1946
7	238005.4	g2526290	1592	1945
7	238005.4	4562130H1	1603	1871
7	238005.4	6093354H1	1607	1891
7	238005.4	2611577H1	1612	1852
7	238005.4	g2003142	1617	1950
7	238005.4	g2213309	1628	1935
7	238005.4	g1893696	1633	1950
7	238005.4	2759732H1	1636	1924
7	238005.4	g4243121	1640	1944
7	238005.4	g4108171	1645	1945
7	238005.4	g3765581	1650	1946
7	238005.4	3027260T6	1677	1899
7	238005.4	1213283H1	1736	1875
7	238005.4	g846910	1739	1936
7	238005.4	2753427H1	1746	1944
7	238005.4	2150089H1	1756	1944
7	238005.4	g1775754	1824	1936
7	238005.4	5288457H1	1860	1978
7	238005.4	g4285357	1863	1946
10	233678.1	1803848F6	1839	2351
10	233678.1	2355751H1	1855	1998
10	233678.1	1923122H1	1895	2186
10	233678.1	1858559H1	1896	2188
10	233678.1	2949425H1	1896	2193
10	233678.1	1858559F6	1896	2326
10	233678.1	4542183H1	1896	2166
10	233678.1	3786222H1	1898	2215
10	233678.1	6091924H1	1943	2212
10	233678.1	4584888H1	1958	2251
10	233678.1	1651475H1	1991	2242
10	233678.1	3967280H1	2006	2242
10	233678.1	5623384H1	2022	2351
10	233678.1	5623284H1	2022	2314
10	233678.1	4948649H1	2031	2325
10	233678.1	1521957H1	2030	2222
10	233678.1	1726538H1	2034	2236

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SEQ ID NO:	Template ID	Component ID	Start	Stop
10	233678.1	1723946H1	2034	2252
10	233678.1	1605814H1	2037	2174
10	233678.1	5554120H1	2037	2321
10	233678.1	1874743F6	2056	2410
10	233678.1	1874743H1	2057	2352
10	233678.1	4857286H1	2097	2352
10	233678.1	1509348H1	2102	2301
10	233678.1	2242767H1	2114	2370
10	233678.1	2848660H1	2126	2428
10	233678.1	2686671H1	2129	2380
10	233678.1	1576284H1	2142	2375
10	233678.1	887052R1	2142	2769
10	233678.1	887052H1	2142	2447
10	233678.1	3785578H1	2149	2345
10	233678.1	3111926H1	2149	2473
10	233678.1	766862H1	2158	2406
10	233678.1	2227686H1	2171	2407
10	233678.1	2013238H1	2171	2277
10	233678.1	823920R1	2176	2721
10	233678.1	1583169H1	2176	2393
10	233678.1	823920H1	2176	2328
10	233678.1	1583137H1	2176	2390
10	233678.1	g1426492	2182	2682
10	233678.1	2275987H1	2225	2478
10	233678.1	4730301H1	2231	2455
10	233678.1	176909H1	2231	2495
10	233678.1	1702710H1	2230	2441
10	233678.1	2845122H1	2231	2466
10	233678.1	5895659H1	2245	2544
10	233678.1	4703091H1	2244	2507
10	233678.1	4214988H1	2256	2569
10	233678.1	1622006H1	2271	2508
10	233678.1	1250975T6	2285	2941
10	233678.1	5874967H1	2299	2566
10	233678.1	3291543H1	2301	2556
10	233678.1	5874909H1	2300	2556
10	233678.1	3291543F6	2301	2749
10	233678.1	4588984H1	2318	2524
10	233678.1	4564713H1	2321	2549
10	233678.1	1607223F6	2329	2631
10	233678.1	1607223H1	2329	2561
10	233678.1	777428R1	2329	2946
10	233678.1	4196029H1	2328	2638
10	233678.1	777428H1	2329	2573
10	233678.1	3098571T6	2334	2942
10	233678.1	2008649H1	2355	2571
10	233678.1	1607223T6	2360	2927
10	233678.1	1803848T6	2373	2929
10	233678.1	2210370T6	2375	2928
10	233678.1	1701603H1	2387	2602



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10	233678.1	5286984H1	2406	2602
10	233678.1	2355751T6	2414	2928
10	233678.1	285554H1	2419	2806
10	233678.1	285554R6	2420	2891
10	233678.1	3289542T6	2435	2926
10	233678.1	2518190T6	2436	2916
10	233678.1	285554T6	2452	2926
10	233678.1	4949921H1	2453	2734
10	233678.1	2814466T6	2451	2932
10	233678.1	3291543T6	2464	2930
10	233678.1	4197595H1	2466	2785
10	233678.1	5105034H1	2468	2740
10	233678.1	g2963989	2472	2972
10	233678.1	g2063675	2474	2971
10	233678.1	g517590	2474	2968
10	233678.1	4950109H1	2484	2768
10	233678.1	1874743T6	2498	2928
10	233678.1	g4107810	2500	2968
10	233678.1	g4175509	2507	2977
10	233678.1	g3411743	2509	2973
10	233678.1	1858559T6	2509	2930
10	233678.1	5848668H1	2517	2804
10	233678.1	g4269824	2519	2968
10	233678.1	3578266T6	2525	2934
10	233678.1	2623196H1	2526	2783
10	233678.1	g3770184	2541	2971
10	233678.1	g3923313	2548	2973
10	233678.1	3274147T6	2551	2910
10	233678.1	g2356198	2568	2897
10	233678.1	1684804T6	2577	2928
10	233678.1	g2784196	2581	2968
10	233678.1	3236618H1	2583	2844
10	233678.1	g2241760	2584	2968
10	233678.1	g316793	2586	2979
10	233678.1	g2816392	2588	2977
10	233678.1	g1954535	2588	2782
10	233678.1	g3700690	2586	2968
10	233678.1	g1887793	2590	2968
10	233678.1	3643238H1	2593	2897
10	233678.1	3650438H1	2594	2894
10	233678.1	2466533H1	2612	2850
10	233678.1	2210370F6	1632	2079
10	233678.1	2210367H1	1632	1886
10	233678.1	4795285H1	1659	1948
10	233678.1	g761628	1670	2092
10	233678.1	3803218H1	1669	1902
10	233678.1	4379275H1	1672	1949
10	233678.1	1606564H1	1682	1912
10	233678.1	5657623H1	1337	1607
10	233678.1	2312430H1	1358	1602

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SEQ ID NO:	Template ID	Component ID	Start	Stop
10	233678.1	3390808H1	1274	1553
10	233678.1	4171822H1	1390	1671
10	233678.1	4508866H1	1437	1711
10	233678.1	4505362H1	1437	1676
10	233678.1	632775H1	1451	1683
10	233678.1	2326782H1	1451	1677
10	233678.1	1684804H1	1281	1507
10	233678.1	1684804F6	1281	1743
10	233678.1	3948184H1	1457	1585
10	233678.1	3804480H1	1487	1706
10	233678.1	5546966H1	1486	1671
10	233678.1	5545829H1	1487	1700
10	233678.1	3088194H1	1498	1735
10	233678.1	3288867H1	1285	1546
10	233678.1	1250975F1	1577	2054
10	233678.1	1250975F6	1577	2051
10	233678.1	5812225H1	1317	1607
10	233678.1	5897556H1	1330	1555
10	233678.1	5893318H1	1330	1640
10	233678.1	5900195H1	1330	1548
10	233678.1	5897434H1	1330	1411
10	233678.1	5898715H1	1330	1634
10	233678.1	1250975H1	1577	1826
10	233678.1	5396479H1	1593	1852
10	233678.1	3797732H1	1604	1709
10	233678.1	5542957H1	1604	1815
10	233678.1	5558368H1	1609	1869
10	233678.1	1002256H1	1	246
10	233678.1	1002256R1	1	482
10	233678.1	1399306H1	74	326
10	233678.1	1399306F6	74	632
10	233678.1	1894055H1	182	422
10	233678.1	1270831F1	393	979
10	233678.1	1270831H1	393	667
10	233678.1	1003419R1	414	915
10	233678.1	1003419H1	414	608
10	233678.1	2518190F6	454	973
10	233678.1	2518190H1	454	706
10	233678.1	5659236H1	474	628
10	233678.1	3288389H1	1161	1413
10	233678.1	5005539H1	1189	1264
10	233678.1	5546782H1	1192	1398
10	233678.1	2809270H1	1239	1456
10	233678.1	2814466H1	762	1073
10	233678.1	5594064H1	496	757
10	233678.1	g764169	598	923
10	233678.1	5546549H1	626	827
10	233678.1	3289542F6	652	1084
10	233678.1	3289542H1	652	893
10	233678.1	5544312H1	687	896

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10	233678.1	2814466F6	762	1334
10	233678.1	2466560H1	766	1014
10	233678.1	2661506H1	769	1035
10	233678.1	2441878H1	884	988
10	233678.1	2437941H1	893	1114
10	233678.1	4785794H1	905	1166
10	233678.1	5574111H1	940	1157
10	233678.1	3964034H1	1006	1286
10	233678.1	4786766H1	1010	1266
10	233678.1	650941H1	1026	1285
10	233678.1	4601334H1	1061	1307
10	233678.1	3275906H1	1100	1352
10	233678.1	3274147F6	1101	1655
10	233678.1	3274147H1	1101	1348
10	233678.1	g3163775	1145	1490
10	233678.1	g4244100	2615	2969
10	233678.1	g4329102	2618	2969
10	233678.1	g4451360	2615	2971
10	233678.1	g3802177	2627	2971
10	233678.1	g3092982	2628	2969
10	233678.1	g3921970	2626	2972
10	233678.1	3516016H1	2646	2918
10	233678.1	1377689H1	2654	2926
10	233678.1	4986111H1	2666	2956
10	233678.1	g845966	2675	2968
10	233678.1	g3777917	2683	2968
10	233678.1	g761629	2702	2962
10	233678.1	g3086793	2719	2968
10	233678.1	5273307H1	2727	2976
10	233678.1	g1139236	2748	2972
10	233678.1	4987591H1	2793	2957
10	233678.1	4987593H1	2794	2968
10	233678.1	2424047H1	2804	2968
10	233678.1	6094676H1	2807	2968
10	233678.1	3083086H1	2842	2968
10	233678.1	3787162H1	2863	2968
10	233678.1	4901030H1	2902	2968
10	233678.1	5103514H1	2904	2974
10	233678.1	5060557H1	1720	2021
10	233678.1	5193174H1	1731	1877
10	233678.1	5003089H1	1750	2052
10	233678.1	3937103H1	1754	2052
10	233678.1	2210665H1	1754	1986
10	233678.1	4951636H2	1754	2033
10	233678.1	4589805H1	1754	1970
10	233678.1	2738891H1	1754	1967
10	233678.1	4793896H1	1754	2005
10	233678.1	1651922H1	1754	1961
10	233678.1	3121552H1	1754	2049
10	233678.1	1236003H1	1754	1975

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10	233678.1	3361752H1	1754	2001
10	233678.1	3282866H1	1754	2003
10	233678.1	3114487H1	1754	2013
10	233678.1	1652026H1	1754	1943
10	233678.1	3804172H1	1809	2145
10	233678.1	g2063923	1820	2231
10	233678.1	5191775H1	1834	1987
10	233678.1	1508970H1	1833	2054
10	233678.1	763920H1	1833	2139
10	233678.1	1803848H1	1839	1940
10	233678.1	2674708H1	1839	2112
11	312243.1	4605386H1	1343	1593
11	312243.1	4605386F7	1343	1784
11	312243.1	1483834H1	1462	1744
11	312243.1	4696494H1	1721	1986
11	312243.1	1823519H1	1734	1955
11	312243.1	1823519F6	1734	2013
11	312243.1	1696230H1	1749	1855
11	312243.1	1695671H1	1757	1986
11	312243.1	1696055H1	1757	1969
11	312243.1	1460083H1	1773	2007
11	312243.1	1823519T6	1787	2427
11	312243.1	3845858H1	1787	1988
11	312243.1	386870H1	1792	2067
11	312243.1	g1515884	1886	2193
11	312243.1	2581154F6	21	197
11	312243.1	2581154H1	21	289
11	312243.1	694181H1	26	222
11	312243.1	3383640H1	33	277
11	312243.1	878997H1	250	387
11	312243.1	881475H1	252	495
11	312243.1	878997R1	252	816
11	312243.1	881475R6	252	705
11	312243.1	641653R6	480	1033
11	312243.1	641653H1	480	729
11	312243.1	g2055007	674	772
11	312243.1	g2063527	684	806
11	312243.1	1316282H1	912	1148
11	312243.1	2120964F6	1099	1478
11	312243.1	2120964H1	1099	1353
11	312243.1	g4186493	1088	1442
11	312243.1	g4451005	1162	1479
11	312243.1	g2273834	1273	1458
11	312243.1	4605366H1	1343	1599
11	312243.1	4605386T7	1923	2422
11	312243.1	641653T6	1947	2441
11	312243.1	2120964T6	1957	2429
11	312243.1	g4305745	2052	2366
11	312243.1	881475T6	2069	2428
11	312243.1	g2138940	2087	2472

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11	312243.1	4601955H1	2114	2269
11	312243.1	g3897513	2313	2467
11	312243.1	g3281088	1	220
11	312243.1	g4189325	1	177
11	312243.1	g3419223	1	219
11	312243.1	3460792H1	1	206
12	425487.3	g4187652	25	467
12	425487.3	g3804148	48	413
12	425487.3	g4265777	50	441
12	425487.3	g1810525	25	243
12	425487.3	g3075563	51	536
12	425487.3	g1576978	582	740
12	425487.3	g2276774	1	236
12	425487.3	g1576933	1	386
12	425487.3	g1860480	1	247
12	425487.3	g3835346	1	498
12	425487.3	g3839178	1	474
12	425487.3	g3839255	1	504
12	425487.3	g1920365	1	417
12	425487.3	g4113340	25	317
12	425487.3	4845742H1	69	296
12	425487.3	g2816806	66	402
12	425487.3	g2881811	75	519
12	425487.3	g2901201	84	461
12	425487.3	g2819800	80	494
12	425487.3	g2899484	91	540
12	425487.3	g2240223	266	660
12	425487.3	5639438H1	531	769
18	222429.3	3135386H1	322	523
18	222429.3	4193895H1	745	1038
18	222429.3	g3917627	750	967
18	222429.3	4773983H1	750	1030
18	222429.3	3110313H1	767	1070
18	222429.3	3818019H1	322	637
18	222429.3	5270075H1	770	1008
18	222429.3	2285210H1	324	583
18	222429.3	3451580H1	783	1039
18	222429.3	5690354H1	788	1045
18	222429.3	3368856H1	323	607
18	222429.3	5692143H1	796	1012
18	222429.3	1695061H1	796	886
18	222429.3	622658H1	803	1062
18	222429.3	4264839H1	814	968
18	222429.3	4382955H1	324	595
18	222429.3	2513281H1	325	584
18	222429.3	g1395660	816	1293
18	222429.3	3182679H1	325	653
18	222429.3	g2881072	817	1302
18	222429.3	1852753H1	328	622
18	222429.3	1852753F6	329	803

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18	222429.3	g4073351	823	1300
18	222429.3	3619548H1	332	599
18	222429.3	1772964H1	829	1110
18	222429.3	5016650H1	835	1123
18	222429.3	891033R1	835	967
18	222429.3	2782761H1	341	620
18	222429.3	4855161H1	347	487
18	222429.3	2802335H1	380	661
18	222429.3	4616684H1	380	653
18	222429.3	g1395574	387	641
18	222429.3	g2032347	393	677
18	222429.3	3553107H1	395	615
18	222429.3	4309262H1	423	747
18	222429.3	4721466T6	428	934
18	222429.3	3466526H1	435	706
18	222429.3	2316414H1	439	697
18	222429.3	g30871119	484	970
18	222429.3	2859627H1	491	779
18	222429.3	g3888504	540	971
18	222429.3	1266732H1	540	774
18	222429.3	g3887405	551	869
18	222429.3	g3801166	560	970
18	222429.3	759622R1	567	804
18	222429.3	759622H1	567	920
18	222429.3	g1496653	586	967
18	222429.3	053812H1	589	802
18	222429.3	1822941H1	602	829
18	222429.3	g3871758	609	967
18	222429.3	g3888431	624	972
18	222429.3	5274484H1	626	878
18	222429.3	g3802660	651	970
18	222429.3	1852753T6	655	1252
18	222429.3	6015049H1	672	969
18	222429.3	1430609H1	686	919
18	222429.3	798615H1	694	975
18	222429.3	3113611T6	698	1224
18	222429.3	1685142H1	742	976
18	222429.3	5900905H1	743	1032
18	222429.3	2078759H1	849	1127
18	222429.3	2043774H1	851	1124
18	222429.3	g2985157	859	1298
18	222429.3	1402308H1	861	1118
18	222429.3	g717242	882	971
18	222429.3	g4109637	883	1293
18	222429.3	g4110383	891	1293
18	222429.3	g3178557	926	1301
18	222429.3	1463294H1	928	1142
18	222429.3	1463335H1	928	1120
18	222429.3	1463294T1	928	1248
18	222429.3	g3871712	929	1292

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18	222429.3	g4307952	950	1295
18	222429.3	1940845T6	953	1251
18	222429.3	1940845R6	953	1294
18	222429.3	1940845H1	953	1181
18	222429.3	3730073H1	957	1278
18	222429.3	g1218940	968	1293
18	222429.3	g2575322	974	1293
18	222429.3	3726214H1	989	1294
18	222429.3	2872559H1	994	1288
18	222429.3	2875466H1	994	1134
18	222429.3	4247311H1	996	1144
18	222429.3	6007354H1	1016	1290
18	222429.3	2154383H1	1040	1293
18	222429.3	5680928H1	1043	1293
18	222429.3	214245H1	1081	1279
18	222429.3	g1069690	1091	1208
18	222429.3	g2212472	1098	1350
18	222429.3	839255H1	1103	1312
18	222429.3	839255R1	1103	1293
18	222429.3	3235786H1	1143	1252
18	222429.3	891762R1	1164	1300
18	222429.3	891762H1	1164	1300
18	222429.3	1849630H1	1184	1293
18	222429.3	1849630F6	1184	1284
18	222429.3	1849630T6	1192	1252
18	222429.3	4865293H1	1210	1293
18	222429.3	4721466F6	1	466
18	222429.3	4721466H1	1	271
18	222429.3	5638504H1	65	317
18	222429.3	5638852H1	65	316
18	222429.3	3076642H1	228	498
18	222429.3	3076642F6	228	487
18	222429.3	4940608H1	247	533
18	222429.3	5388153H1	267	412
18	222429.3	3750219H1	253	506
18	222429.3	3937336H1	253	527
18	222429.3	4122661H1	253	500
18	222429.3	3680329H1	253	572
18	222429.3	4943143H1	253	516
18	222429.3	4585314H1	254	510
18	222429.3	2732104H1	254	510
18	222429.3	2537505H1	257	492
18	222429.3	g2015403	274	591
18	222429.3	4786776H1	274	526
18	222429.3	4045742H1	274	579
18	222429.3	3567962H1	274	529
18	222429.3	1386440H1	276	413
18	222429.3	5187636H1	275	560
18	222429.3	5197384H1	277	547
18	222429.3	1572627H1	277	491

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18	222429.3	1572748H1	277	502
18	222429.3	2372113H1	279	527
18	222429.3	5187033H1	279	420
18	222429.3	2785651H1	279	563
18	222429.3	2727323H1	280	534
18	222429.3	3134034H1	280	554
18	222429.3	927455H1	280	558
18	222429.3	927455R1	280	894
18	222429.3	2596333H1	280	528
18	222429.3	2372113F6	280	797
18	222429.3	5847736H1	281	565
18	222429.3	2853635H1	281	538
18	222429.3	2854289H1	281	561
18	222429.3	2730059H1	281	540
18	222429.3	1268178F1	282	704
18	222429.3	2492274H1	282	517
18	222429.3	4124267H1	282	513
18	222429.3	2459370H1	282	511
18	222429.3	1268178H1	282	556
18	222429.3	6013641H1	282	518
18	222429.3	5378585H1	283	535
18	222429.3	3985882H1	274	384
18	222429.3	3870288H1	285	576
18	222429.3	4608143H1	283	534
18	222429.3	3983482H1	286	464
18	222429.3	5843644H1	286	511
18	222429.3	2605545H1	288	536
18	222429.3	3088525H1	288	577
18	222429.3	3218430H1	290	587
18	222429.3	g1496652	291	538
18	222429.3	4248167H1	294	568
18	222429.3	4767534H1	294	424
18	222429.3	4174335H1	294	613
18	222429.3	3695889H1	295	582
18	222429.3	5592876H1	294	443
18	222429.3	2658509H1	296	546
18	222429.3	730756H1	296	565
18	222429.3	4613408H1	297	505
18	222429.3	264145H1	297	616
18	222429.3	5810372H1	299	665
18	222429.3	3319788H1	300	476
18	222429.3	4202422H1	301	596
18	222429.3	4613487H1	306	507
18	222429.3	g712550	306	583
18	222429.3	5090055H1	307	567
18	222429.3	2823320H1	309	631
18	222429.3	2529472H1	311	578
18	222429.3	4850016H1	312	590
18	222429.3	4606729H1	313	569
18	222429.3	4608174H1	313	567



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18	222429.3	4334782H1	314	584
18	222429.3	3508043H1	314	609
18	222429.3	4638791H1	316	578
18	222429.3	2941851H1	316	594
18	222429.3	2533245H1	317	556
18	222429.3	033345H1	317	444
18	222429.3	2888083H1	317	573
18	222429.3	2766782H1	318	591
18	222429.3	g1069689	321	669
18	222429.3	2514805H1	321	656
19	366739.2	3793584H1	1	276
19	366739.2	3093259F6	1	396
19	366739.2	3093259H1	1	272
19	366739.2	5586972H1	28	288
19	366739.2	1562228H1	28	244
19	366739.2	g1976820	31	385
19	366739.2	5472436H1	30	254
19	366739.2	g1974601	38	290
19	366739.2	3536311H1	63	278
19	366739.2	g1301142	203	492
19	366739.2	2996019H1	226	495
19	366739.2	1291844F6	265	754
19	366739.2	1291844F1	265	757
19	366739.2	1291844H1	265	495
19	366739.2	5107426H1	323	395
19	366739.2	6077830H1	331	653
19	366739.2	2690675H1	335	549
19	366739.2	3673120H1	341	654
19	366739.2	4626753H1	343	626
19	366739.2	2814563H1	344	559
19	366739.2	5019593H1	348	607
19	366739.2	5591354H1	350	602
19	366739.2	4337887H1	348	533
19	366739.2	2830045H1	350	618
19	366739.2	3614922H1	351	637
19	366739.2	3034106H1	352	640
19	366739.2	4765938H1	352	635
19	366739.2	3375151H1	354	621
19	366739.2	804576H1	357	612
19	366739.2	2188984H1	360	622
19	366739.2	157111H1	361	569
19	366739.2	5391316H1	370	647
19	366739.2	808513H1	372	654
19	366739.2	1966930H1	381	617
19	366739.2	1966930R6	381	755
19	366739.2	5194739H1	431	658
19	366739.2	4946548H1	431	604
19	366739.2	4768533F6	455	865
19	366739.2	4768533H1	455	739
19	366739.2	g778805	457	743

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SEQ ID NO:	Template ID	Component ID	Start	Stop
19	366739.2	g1921131	463	977
19	366739.2	4353206H2	492	757
19	366739.2	5325529H1	501	794
19	366739.2	5322285H1	501	774
19	366739.2	5322857H1	502	752
19	366739.2	869959H1	564	808
19	366739.2	875716R6	564	1081
19	366739.2	875716H1	564	855
19	366739.2	875716R1	564	1203
19	366739.2	3221359H1	601	939
19	366739.2	3869036H1	630	910
19	366739.2	3353207H1	645	955
19	366739.2	g2016781	644	916
19	366739.2	4587185H1	645	907
19	366739.2	5406614H1	653	850
19	366739.2	g1300754	650	1123
19	366739.2	370417H1	713	1027
19	366739.2	1265285H1	720	861
19	366739.2	1265285R1	720	1186
19	366739.2	1265102H1	720	891
19	366739.2	3946741H1	742	1024
19	366739.2	3603130H1	771	1093
19	366739.2	5472209H1	846	1043
19	366739.2	3717241H1	870	1172
19	366739.2	1291844T6	871	1416
19	366739.2	2608544H1	898	1103
19	366739.2	g922177	903	1148
19	366739.2	251965H1	925	1273
19	366739.2	3470188H1	935	1210
19	366739.2	g2838122	936	1451
19	366739.2	1636138F6	963	1441
19	366739.2	1636138H1	963	1184
19	366739.2	923492H1	968	1270
19	366739.2	3811371H1	977	1294
19	366739.2	g3430481	978	1454
19	366739.2	g4282887	982	1458
19	366739.2	g4372283	986	1454
19	366739.2	5138130H1	1015	1307
19	366739.2	g2958044	1019	1453
19	366739.2	1636138T6	1035	1416
19	366739.2	g2397938	1039	1452
19	366739.2	1415729H1	1048	1289
19	366739.2	676581H1	1055	1273
19	366739.2	3093259T6	1056	1412
19	366739.2	g1921132	1059	1465
19	366739.2	337785H1	1075	1288
19	366739.2	3773169H1	1088	1367
19	366739.2	g2820846	1095	1453
19	366739.2	g2322961	1109	1457
19	366739.2	g3923575	1124	1453

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SEQ ID NO:	Template ID	Component ID	Start	Stop
19	366739.2	5399095H1	1135	1273
19	366739.2	g1266798	1140	1459
19	366739.2	g2883870	1143	1434
19	366739.2	g4325983	1152	1458
19	366739.2	g4325893	1154	1458
19	366739.2	1223335T1	1154	1414
19	366739.2	1223335H1	1154	1434
19	366739.2	g3959938	1177	1453
19	366739.2	g1970868	1179	1468
19	366739.2	2228180H1	1185	1460
19	366739.2	g2574692	1223	1462
19	366739.2	2505795H1	1237	1456
19	366739.2	g2877025	1242	1453
19	366739.2	g4268440	1259	1453
19	366739.2	g2575341	1264	1453
19	366739.2	g778806	1268	1452
19	366739.2	1772854R6	1273	1453
19	366739.2	1772849H1	1273	1453
19	366739.2	g1264282	1343	1473
20	474635.6	340786H1	2181	2422
20	474635.6	2242239H1	2179	2347
20	474635.6	g991123	2179	2514
20	474635.6	2234442H1	2179	2397
20	474635.6	g1056895	2180	2498
20	474635.6	g959046	2182	2471
20	474635.6	g1004715	2183	2527
20	474635.6	4996329T6	2189	2625
20	474635.6	4746496H1	2192	2451
20	474635.6	g3737429	2197	2728
20	474635.6	g1039999	2210	2512
20	474635.6	g1081479	2210	2391
20	474635.6	2204795T6	2217	2671
20	474635.6	g3736649	2220	2704
20	474635.6	2242239T6	2220	2816
20	474635.6	3733918T6	2230	2786
20	474635.6	g3742588	2229	2728
20	474635.6	g1014336	2235	2566
20	474635.6	3638137H1	2230	2511
20	474635.6	g835640	2232	2567
20	474635.6	1666516H1	2236	2449
20	474635.6	2083270H1	2262	2567
20	474635.6	1761002H1	2284	2581
20	474635.6	612373H1	2291	2581
20	474635.6	665014H1	2291	2558
20	474635.6	g867547	2291	2662
20	474635.6	g1986029	2312	2772
20	474635.6	3000193H1	2318	2627
20	474635.6	461079H1	2328	2588
20	474635.6	g1274411	2332	2863
20	474635.6	2237019H1	2332	2557

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SEQ ID NO:	Template ID	Component ID	Start	Stop
20	474635.6	g856812	2334	2693
20	474635.6	5275784H1	2343	2627
20	474635.6	4085512H1	2393	2714
20	474635.6	g1240371	2407	2873
20	474635.6	g848488	2408	2767
20	474635.6	g897948	2408	2722
20	474635.6	g848512	2408	2850
20	474635.6	g1444420	2410	2880
20	474635.6	665169H1	2419	2677
20	474635.6	2090332H1	2439	2728
20	474635.6	g1004911	2454	2877
20	474635.6	g2900276	2464	2950
20	474635.6	g3848664	2464	2910
20	474635.6	5432669H1	2472	2741
20	474635.6	3134278H1	2486	2792
20	474635.6	g4174578	2487	2999
20	474635.6	g3433111	2489	2991
20	474635.6	g3419207	2491	3017
20	474635.6	4087752H1	2502	2814
20	474635.6	5327883H1	2520	2806
20	474635.6	5326114H1	2523	2818
20	474635.6	5326214H1	2524	2806
20	474635.6	g3229542	2527	3017
20	474635.6	2757338F6	2528	3008
20	474635.6	g3405868	938	1282
20	474635.6	572947H1	944	1190
20	474635.6	3002964H1	961	1013
20	474635.6	g982216	976	1338
20	474635.6	2309094H1	1029	1217
20	474635.6	4638158H1	1036	1287
20	474635.6	1912857H1	1042	1285
20	474635.6	g916500	1047	1247
20	474635.6	3633880H1	1055	1354
20	474635.6	3634680H1	1055	1325
20	474635.6	4546165H1	1074	1351
20	474635.6	g1276377	1073	1325
20	474635.6	5677925H1	1154	1382
20	474635.6	4663039H1	1163	1425
20	474635.6	3511926H1	1174	1426
20	474635.6	4996329H1	1324	1605
20	474635.6	4996329F6	1324	1683
20	474635.6	4740909H1	1330	1603
20	474635.6	647910H1	1344	1623
20	474635.6	1715955F6	1431	1896
20	474635.6	3281456H1	1463	1723
20	474635.6	2204795F6	1494	2015
20	474635.6	2204795H1	1494	1739
20	474635.6	4546440H1	1504	1781
20	474635.6	1669175H1	1524	1751
20	474635.6	1443714F6	1644	2033

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SEQ ID NO:	Template ID	Component ID	Start	Stop
20	474635.6	1443714H1	1644	1900
20	474635.6	1715955H1	1680	1896
20	474635.6	4088721H1	1699	1960
20	474635.6	4049359H1	1718	1989
20	474635.6	3322874H1	1720	1987
20	474635.6	3733918F6	1806	2215
20	474635.6	4411044H1	1832	2038
20	474635.6	5173224H1	1866	2144
20	474635.6	5098372H1	1930	2150
20	474635.6	4412048H1	1933	2137
20	474635.6	1265323R1	1935	2488
20	474635.6	1265323H1	1935	2151
20	474635.6	376565H1	1961	2151
20	474635.6	1630741H1	1961	2147
20	474635.6	1631939H1	1961	2151
20	474635.6	6102550H1	1966	2151
20	474635.6	6102850H1	1966	2154
20	474635.6	g958816	2172	2378
20	474635.6	g1047675	2173	2505
20	474635.6	g965279	2173	2563
20	474635.6	g3098969	2173	2510
20	474635.6	g1202194	2174	2528
20	474635.6	g1099631	2174	2567
20	474635.6	g1047662	2174	2514
20	474635.6	g1228299	2176	2306
20	474635.6	g1193583	2176	2464
20	474635.6	g2912515	2176	2667
20	474635.6	2242239F6	2176	2646
20	474635.6	g1211570	2176	2487
20	474635.6	g1225734	2176	2491
20	474635.6	g1226785	2176	2462
20	474635.6	g1043793	2176	2392
20	474635.6	g1162551	2176	2505
20	474635.6	1725002H1	1	207
20	474635.6	1726247H1	1	216
20	474635.6	3495066H1	1	301
20	474635.6	1727369F6	1	387
20	474635.6	1727369H1	1	249
20	474635.6	3109953H1	10	305
20	474635.6	2362517H1	16	279
20	474635.6	2361835H1	16	270
20	474635.6	3617437H1	58	382
20	474635.6	2659652H1	91	348
20	474635.6	3734189H1	92	393
20	474635.6	2679260H1	96	409
20	474635.6	1466462H1	96	295
20	474635.6	3563056H1	96	415
20	474635.6	2235355H1	100	364
20	474635.6	2202076H1	99	367
20	474635.6	3129502H1	101	436

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SEQ ID NO:	Template ID	Component ID	Start	Stop
20	474635.6	2051824H1	118	293
20	474635.6	3633032H1	122	294
20	474635.6	3150266H1	126	379
20	474635.6	2051825H1	132	414
20	474635.6	g1227685	128	482
20	474635.6	4288817H1	146	437
20	474635.6	4860794H1	155	246
20	474635.6	174879H1	161	364
20	474635.6	5700836H1	163	471
20	474635.6	5539631H2	182	413
20	474635.6	5206045H1	220	491
20	474635.6	375911R6	244	566
20	474635.6	375911H1	244	536
20	474635.6	g657023	255	500
20	474635.6	3939757H1	275	433
20	474635.6	3939749H1	276	433
20	474635.6	g704964	290	589
20	474635.6	g1013184	290	695
20	474635.6	g1472478	326	795
20	474635.6	1220725H1	355	600
20	474635.6	4980839H1	358	659
20	474635.6	375911T6	370	902
20	474635.6	3675542H1	371	682
20	474635.6	3669542H1	371	695
20	474635.6	3671542H1	371	558
20	474635.6	g1447913	383	835
20	474635.6	1282181H1	391	534
20	474635.6	g2020599	418	783
20	474635.6	4133026H2	447	725
20	474635.6	2236848H1	450	698
20	474635.6	g1278197	473	1017
20	474635.6	g1941907	514	972
20	474635.6	g2695124	514	1013
20	474635.6	1470350F6	554	998
20	474635.6	578500H1	557	758
20	474635.6	g4371917	558	1013
20	474635.6	g1447816	558	1013
20	474635.6	g2702599	559	1013
20	474635.6	505476H1	560	793
20	474635.6	2086083H1	579	884
20	474635.6	g3430549	602	1020
20	474635.6	g1671099	609	1029
20	474635.6	g1472421	658	1013
20	474635.6	g656857	700	1013
20	474635.6	3381660H1	715	902
20	474635.6	4270822H1	742	1016
20	474635.6	g1977606	753	1013
20	474635.6	4594512H1	755	1013
20	474635.6	g944107	761	1016
20	474635.6	1470350H1	802	998

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SEQ ID NO:	Template ID	Component ID	Start	Stop
20	474635.6	236251716	802	970
20	474635.6	g1011912	812	1004
20	474635.6	g1201979	871	1013
20	474635.6	4412657H1	883	969
20	474635.6	g916501	928	1234
20	474635.6	g1238628	931	1126
20	474635.6	g2057226	934	1034
20	474635.6	3450474H1	938	1128
20	474635.6	g2022938	938	1147
20	474635.6	g982171	939	1266
20	474635.6	2757338H1	2528	2825
20	474635.6	2757338R6	2528	2995
20	474635.6	5425690H1	2531	2796
20	474635.6	g1046524	2535	2900
20	474635.6	g3134715	2534	2985
20	474635.6	380176H1	2537	2654
20	474635.6	g1186355	2560	2772
20	474635.6	g856701	2582	2772
20	474635.6	1795930H1	2583	2701
20	474635.6	g651862	2589	2849
20	474635.6	g651879	2589	2896
20	474635.6	g3076096	2594	2992
20	474635.6	g4078849	2605	2999
20	474635.6	g835594	2606	2985
20	474635.6	g1081771	2618	2893
20	474635.6	g1266372	2625	2900
20	474635.6	971739H1	2631	2952
20	474635.6	1808492H1	2639	2869
20	474635.6	649261H1	2645	2940
20	474635.6	g867525	2652	2949
20	474635.6	1352072H1	2682	2960
20	474635.6	g848398	2688	2993
20	474635.6	g3754493	2700	2999
20	474635.6	g2839098	2719	3187
20	474635.6	g848419	2728	3010
20	474635.6	g1524564	2740	3015
20	474635.6	g806064	2765	2972
20	474635.6	1402667H1	2791	3002
20	474635.6	g2752910	2841	2938
20	474635.6	g715956	2849	2968
20	474635.6	g3428030	2948	2997
24	411449.2	3292975H1	654	917
24	411449.2	g1277242	663	1052
24	411449.2	1355750H1	662	944
24	411449.2	1355750F6	662	1150
24	411449.2	g1443735	707	1043
24	411449.2	1984010H1	722	990
24	411449.2	g1243124	747	1012
24	411449.2	g1243130	747	939
24	411449.2	g1243104	748	938

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SEQ ID NO:	Template ID	Component ID	Start	Stop
24	411449.2	g1243131	773	923
24	411449.2	g841841	823	1176
24	411449.2	g1635822	830	1026
24	411449.2	677686H1	890	1140
24	411449.2	2896619H1	891	1077
24	411449.2	g3933055	644	1016
24	411449.2	1689376H1	898	1105
24	411449.2	3783111H1	908	1221
24	411449.2	4796764H1	515	795
24	411449.2	4796772H1	515	795
24	411449.2	g2037311	533	814
24	411449.2	g2819773	551	815
24	411449.2	057539H1	507	708
24	411449.2	g3098856	560	922
24	411449.2	g1125331	560	950
24	411449.2	g1386245	583	970
24	411449.2	3323088H1	586	854
24	411449.2	g2816548	607	1022
24	411449.2	3660962H1	612	867
24	411449.2	g1887649	615	946
24	411449.2	g1489541	618	866
24	411449.2	g1489523	619	965
24	411449.2	g4327095	909	1337
24	411449.2	5275975H1	911	1077
24	411449.2	2682468H1	941	1157
24	411449.2	2682429H1	943	1218
24	411449.2	3448516T6	966	1496
24	411449.2	5517642H1	966	1232
24	411449.2	3723582H1	970	1267
24	411449.2	589712H1	978	1222
24	411449.2	589712R1	978	1519
24	411449.2	2613128H1	1015	1252
24	411449.2	1355750T6	1052	1482
24	411449.2	3820979H1	1057	1340
24	411449.2	1811236H1	1068	1326
24	411449.2	1811236F6	1068	1562
24	411449.2	1811236T6	1083	1726
24	411449.2	g4033830	1106	1519
24	411449.2	g3917075	1106	1519
24	411449.2	g3917072	1108	1519
24	411449.2	3297093H1	1117	1204
24	411449.2	4513565H1	1174	1440
24	411449.2	4798512H1	1190	1460
24	411449.2	2645110H1	1195	1450
24	411449.2	2096680R6	1216	1644
24	411449.2	2096680H1	1216	1462
24	411449.2	321613H1	1233	1492
24	411449.2	2995437H1	1251	1538
24	411449.2	g4371719	1378	1775
24	411449.2	2096680T6	1380	1715



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SEQ ID NO:	Template ID	Component ID	Start	Stop
24	411449.2	4653462H1	1411	1685
24	411449.2	g1489542	1416	1765
24	411449.2	4144044H1	1422	1709
24	411449.2	g1224160	1425	1770
24	411449.2	g1224144	1433	1770
24	411449.2	g1489524	1473	1766
24	411449.2	g769281	1485	1823
24	411449.2	g2955239	1501	1777
24	411449.2	g4078417	1503	1770
24	411449.2	g1224166	1517	1770
24	411449.2	g1224143	1529	1770
24	411449.2	1972386H1	1531	1804
24	411449.2	g4084798	1534	1954
24	411449.2	g1224165	1560	1770
24	411449.2	g784201	1564	1798
24	411449.2	g2657184	1564	1869
24	411449.2	5919979H1	1256	1559
24	411449.2	008248H1	1258	1569
24	411449.2	g889333	1271	1666
24	411449.2	g1139751	1287	1746
24	411449.2	589712F1	1305	1770
24	411449.2	3659604H1	1316	1588
24	411449.2	2598057H1	1327	1458
24	411449.2	g3178153	1345	1772
24	411449.2	g4187548	1373	1774
24	411449.2	g574838	1617	1824
24	411449.2	3778628H1	1645	1948
24	411449.2	g3785651	1656	1770
24	411449.2	763788H1	1696	1765
24	411449.2	1490003H1	1788	2054
24	411449.2	g2000780	1844	2185
24	411449.2	4704775H1	1862	2105
24	411449.2	g3921575	1873	2274
24	411449.2	g3231159	1901	2276
24	411449.2	g2222962	1902	2260
24	411449.2	g2222973	1902	2258
24	411449.2	638241H1	1908	2160
24	411449.2	001302H1	1908	2236
24	411449.2	1625423F6	1911	2197
24	411449.2	1625423H1	1911	2113
24	411449.2	491356H1	1923	2185
24	411449.2	g1964447	1927	2193
24	411449.2	g1963729	1927	2263
24	411449.2	g1379590	1936	2272
24	411449.2	g1472323	1948	2197
24	411449.2	2399751H1	1951	2192
24	411449.2	4856606H1	1973	2197
24	411449.2	995171H1	1978	2209
24	411449.2	g1472316	1979	2197
24	411449.2	5194364H2	1982	2194

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SEQ ID NO:	Template ID	Component ID	Start	Stop
24	411449.2	3451002H1	2043	2168
24	411449.2	g4524789	2083	2553
24	411449.2	g2159625	2209	2405
24	411449.2	g2154000	2210	2639
24	411449.2	g3739242	2216	2561
24	411449.2	5276078H1	2220	2494
24	411449.2	g889241	2223	2564
24	411449.2	g1148058	2235	2554
24	411449.2	g3077322	2268	2561
24	411449.2	g784419	2295	2555
24	411449.2	2758260H1	2312	2541
24	411449.2	g4109094	2349	2619
24	411449.2	g1785173	2442	2554
24	411449.2	3504710H1	2444	2554
24	411449.2	g3678027	2544	2899
24	411449.2	g3412805	2545	2944
24	411449.2	g3214749	2546	2950
24	411449.2	g4149617	2546	2995
24	411449.2	g2969810	2546	2703
24	411449.2	g746614	2551	2916
24	411449.2	g3756632	2550	2615
24	411449.2	g2969693	2551	2970
24	411449.2	g3770455	2552	2832
24	411449.2	g2538262	2552	2618
24	411449.2	g2882695	2552	2871
24	411449.2	3073355H1	3031	3337
24	411449.2	2502022H1	3034	3299
24	411449.2	2203860H1	3035	3321
24	411449.2	2415335H1	3040	3241
24	411449.2	4068029H1	3043	3337
24	411449.2	3633303H1	3044	3319
24	411449.2	1972353H1	3039	3301
24	411449.2	g746725	3044	3326
24	411449.2	3737404H1	3044	3324
24	411449.2	3557887H1	3044	3317
24	411449.2	3295556H1	3046	3325
24	411449.2	4114476H1	3047	3337
24	411449.2	4795315H1	3048	3337
24	411449.2	577995H1	3047	3307
24	411449.2	3167827H1	3051	3339
24	411449.2	5686359H1	3055	3339
24	411449.2	2781948H1	3066	3331
24	411449.2	2561784H2	3068	3340
24	411449.2	4712882H1	3077	3339
24	411449.2	5374882H1	3086	3335
24	411449.2	g2153894	3087	3334
24	411449.2	3596863H1	3133	3337
24	411449.2	4675071H1	3155	3312
24	411449.2	5285819H1	3161	3337
24	411449.2	3638749H1	3186	3301

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SEQ ID NO:	Template ID	Component ID	Start	Stop
24	411449.2	2707450H1	3198	3319
24	411449.2	5695240H1	3208	3337
24	411449.2	5059716H1	3251	3316
24	411449.2	g1781709	1	493
24	411449.2	g1775656	1	432
24	411449.2	2472307F6	18	397
24	411449.2	2472307H1	18	265
24	411449.2	g822142	36	381
24	411449.2	g2000779	51	258
24	411449.2	2782774H1	79	356
24	411449.2	g1295851	138	810
24	411449.2	g1312393	138	614
24	411449.2	3448516R6	142	624
24	411449.2	g1010505	2552	2714
24	411449.2	g1692011	2553	2956
24	411449.2	1274760F1	2555	2848
24	411449.2	g2658624	2554	2974
24	411449.2	g1921464	2554	2863
24	411449.2	g1153184	2554	2764
24	411449.2	g2768980	2555	2668
24	411449.2	g2153787	2555	2972
24	411449.2	g3117405	2555	2850
24	411449.2	g2933847	2554	2995
24	411449.2	g2934183	2555	3034
24	411449.2	g2568950	2556	2868
24	411449.2	2018010H1	2562	2849
24	411449.2	g1636435	2656	2796
24	411449.2	2050014H1	2689	2969
24	411449.2	2910217H1	2694	2967
24	411449.2	g2153999	2709	3188
24	411449.2	g2204848	2778	3188
24	411449.2	2476089H1	2798	3037
24	411449.2	g1925326	2847	3323
24	411449.2	g1678125	2913	3317
24	411449.2	g2558304	2937	3101
24	411449.2	g2100182	2951	3334
24	411449.2	506236H1	2971	3182
24	411449.2	3518329H1	2976	3311
24	411449.2	3473456H1	2979	3337
24	411449.2	1698871H1	2986	3230
24	411449.2	4512967H1	2987	3259
24	411449.2	g1692106	2999	3198
24	411449.2	3081295H1	3009	3317
24	411449.2	4819058H1	3020	3324
24	411449.2	3492756H1	3028	3337
24	411449.2	3448616H1	142	413
24	411449.2	6138342H1	150	451
24	411449.2	1957671H1	154	438
24	411449.2	g3888550	166	634
24	411449.2	239023H1	199	433

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SEQ ID NO:	Template ID	Component ID	Start	Stop
24	411449.2	463172H1	205	453
24	411449.2	g1471862	257	732
24	411449.2	g1471855	257	691
24	411449.2	5153884H1	352	608
24	411449.2	593640H1	385	596
24	411449.2	g2037867	408	741
24	411449.2	g2946071	438	791
25	18549.2	2666675H1	1	256
25	18549.2	2666675F6	1	447
25	18549.2	2872055H1	35	324
25	18549.2	3674803H1	45	340
25	18549.2	g4393247	48	393
26	236043.3	4854788H1	57	322
26	236043.3	2637174H1	57	313
26	236043.3	4154039H1	57	326
26	236043.3	4154437H1	66	332
26	236043.3	g1784274	72	327
26	236043.3	g1782229	74	421
26	236043.3	5167751H1	1	259
26	236043.3	4154654H1	31	288
26	236043.3	2637174F6	57	407
26	236043.3	5856494H1	52	322
26	236043.3	753667H1	57	311
26	236043.3	2638855F6	615	912
26	236043.3	2638855T6	624	1270
26	236043.3	4000427H1	750	952
26	236043.3	118534H1	826	1086
26	236043.3	g1782014	858	1286
26	236043.3	g2553565	891	1313
26	236043.3	g707651	915	1263
26	236043.3	506959H1	946	1251
26	236043.3	g1792156	953	1283
26	236043.3	g2616478	957	1266
26	236043.3	g1792664	975	1281
26	236043.3	g3754889	1007	1313
26	236043.3	g707650	1011	1322
26	236043.3	g1784007	1062	1313
26	236043.3	g1782018	1086	1313
26	236043.3	1209542H1	1096	1326
26	236043.3	2433413H1	1134	1379
26	236043.3	g1792600	1184	1283
26	236043.3	440101976	1210	1699
26	236043.3	2637174T6	1202	1260
26	236043.3	g2342125	1329	1530
26	236043.3	118858H1	75	275
26	236043.3	g1760400	77	392
26	236043.3	g1664195	79	448
26	236043.3	g1784031	82	542
26	236043.3	5298110H1	93	360
26	236043.3	5280294H1	110	358

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SEQ ID NO:	Template ID	Component ID	Start	Stop
26	236043.3	3516062H1	113	391
26	236043.3	4012792H1	114	402
26	236043.3	5856458H1	176	453
26	236043.3	5856757H1	176	310
26	236043.3	g1784279	204	475
26	236043.3	g707758	277	621
26	236043.3	4150892H1	309	572
26	236043.3	g1792258	414	803
26	236043.3	3420770H1	432	622
26	236043.3	2638855H1	615	846
26	236043.3	121222H1	615	730
27	445433.2	3287379H1	1	244
27	445433.2	2275475H1	193	424
27	445433.2	3967290T6	308	705
27	445433.2	3967290F6	315	723
27	445433.2	4133307H1	315	574
27	445433.2	3967290H1	315	558
27	445433.2	1343762F6	681	1042
29	257121.2	2808987H1	805	1039
29	257121.2	4021166H1	831	1064
29	257121.2	g1720349	2858	3353
29	257121.2	g1522235	2858	3196
29	257121.2	3517880H1	2876	3155
29	257121.2	4459330H1	2876	3127
29	257121.2	3917168H1	2886	3150
29	257121.2	3160987H1	2897	3193
29	257121.2	4506429H1	2901	2981
29	257121.2	4624935H1	2909	3181
29	257121.2	879657H1	3382	3640
29	257121.2	434236H1	3388	3610
29	257121.2	2078370H1	2935	3207
29	257121.2	5058836H1	2958	3241
29	257121.2	1955941H1	2984	3250
29	257121.2	3993470H1	3015	3322
29	257121.2	2693754H1	3017	3296
29	257121.2	3528783H1	3020	3325
29	257121.2	2945721H1	3030	3196
29	257121.2	2947643H1	3028	3355
29	257121.2	5271360H1	3029	3276
29	257121.2	g3840958	3443	3849
29	257121.2	406453H1	3445	3689
29	257121.2	865275H1	3451	3684
29	257121.2	2373951H1	3455	3695
29	257121.2	g2329311	3465	3849
29	257121.2	5201726T6	3463	3823
29	257121.2	3929807H1	3466	3775
29	257121.2	4466537H1	3466	3723
29	257121.2	2071630H1	2781	3036
29	257121.2	2714703H1	2800	3056
29	257121.2	630218H1	2804	3049

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SEQ ID NO:	Template ID	Component ID	Start	Stop
29	257121.2	g2219708	2818	3192
29	257121.2	5435973H1	2403	2648
29	257121.2	g2189770	2409	2575
29	257121.2	3091740F6	2434	2892
29	257121.2	3091740H1	2435	2721
29	257121.2	4931375H1	2450	2729
29	257121.2	5086828H1	2466	2717
29	257121.2	4706266H1	2474	2701
29	257121.2	4623814H1	2489	2746
29	257121.2	1711729H1	2493	2682
29	257121.2	3808352H1	2493	2707
29	257121.2	g1999011	2496	2841
29	257121.2	2080982H1	2500	2772
29	257121.2	4936350H1	2507	2800
29	257121.2	2473816H1	2534	2761
29	257121.2	2473816F6	2534	3077
29	257121.2	2228396H1	2540	2791
29	257121.2	4021166F6	831	1284
29	257121.2	2051255H1	838	1128
29	257121.2	3001820F6	870	1041
29	257121.2	3001820H1	871	1152
29	257121.2	5395159H1	935	1013
29	257121.2	492727H1	1100	1333
29	257121.2	2738108H1	1122	1378
29	257121.2	3344641H1	1132	1370
29	257121.2	526778H1	1241	1504
29	257121.2	310738H1	1296	1527
29	257121.2	2754594H1	1296	1556
29	257121.2	4056308H1	3034	3328
29	257121.2	4021166T6	3036	3558
29	257121.2	4465912H1	3059	3327
29	257121.2	5586546H1	3067	3312
29	257121.2	g1239705	3068	3332
29	257121.2	552938H1	3096	3365
29	257121.2	3730966H1	3111	3436
29	257121.2	g1444147	3121	3612
29	257121.2	5550566H1	3172	3413
29	257121.2	5507424H1	1840	2028
29	257121.2	5508405H1	1840	2087
29	257121.2	3000303H1	1849	2065
29	257121.2	g1975045	1853	2120
29	257121.2	5662088H1	1854	2082
29	257121.2	g3770956	1868	2224
29	257121.2	g3802745	1899	2216
29	257121.2	4892961H1	1903	2189
29	257121.2	3479021H1	1924	2149
29	257121.2	1690528H1	1938	2158
29	257121.2	4467223H1	1953	2159
29	257121.2	5270694H1	3395	3651
29	257121.2	g1719483	3568	3855

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SEQ ID NO:	Template ID	Component ID	Start	Stop
29	257121.2	g4531585	3586	3849
29	257121.2	3720345H1	3594	3759
29	257121.2	25669980H1	3601	3849
29	257121.2	g1203034	3610	3783
29	257121.2	g4450994	3614	3849
29	257121.2	4901641H1	3614	3822
29	257121.2	4960977H1	2828	3096
29	257121.2	g2728590	2834	3223
29	257121.2	3162492H1	2842	3125
29	257121.2	g1719482	2858	3289
29	257121.2	g2211339	3417	3848
29	257121.2	g2659436	3422	3854
29	257121.2	g4296041	3428	3849
29	257121.2	5921004H1	3429	3679
29	257121.2	2962704H1	3431	3741
29	257121.2	5922274H1	3431	3715
29	257121.2	g2329245	3433	3703
29	257121.2	g4328182	3435	3849
29	257121.2	1994936R6	3438	3848
29	257121.2	1994936T6	3438	3808
29	257121.2	1994936H1	3438	3705
29	257121.2	865275T1	3439	3794
29	257121.2	453069H1	1980	2189
29	257121.2	5982875H1	2023	2298
29	257121.2	g2457731	2054	2450
29	257121.2	2883663H1	2077	2328
29	257121.2	1786163H1	2091	2273
29	257121.2	3433528H1	2097	2303
29	257121.2	2588747H2	2117	2375
29	257121.2	4715351H1	2157	2241
29	257121.2	4691328H1	2198	2462
29	257121.2	2904069H1	2226	2537
29	257121.2	4300967H1	2236	2508
29	257121.2	4624918H1	2909	3183
29	257121.2	2374252H1	2910	3120
29	257121.2	4909794H1	2922	3203
29	257121.2	1741105H1	2925	3159
29	257121.2	1741564H1	2925	3086
29	257121.2	1741564R6	2925	3384
29	257121.2	g531805	3303	3701
29	257121.2	3091740T6	3306	3809
29	257121.2	2697055H1	3308	3597
29	257121.2	1672059T6	3344	3809
29	257121.2	g2818402	3359	3858
29	257121.2	2423136H1	3377	3613
29	257121.2	879657T1	3382	3809
29	257121.2	g4329746	3472	3849
29	257121.2	g4243717	3477	3856
29	257121.2	3929886H1	3478	3759
29	257121.2	g2619701	3487	3892

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SEQ ID NO:	Template ID	Component ID	Start	Stop
29	257121.2	g2835679	3490	3849
29	257121.2	g2216408	3495	3849
29	257121.2	3040078H1	3500	3780
29	257121.2	g2261946	3498	3849
29	257121.2	g1522116	3502	3855
29	257121.2	g3190675	3510	3848
29	257121.2	g3281587	3517	3852
29	257121.2	g1186385	3517	3849
29	257121.2	g3069539	3519	3856
29	257121.2	g3229168	3523	3849
29	257121.2	g2278678	3527	3855
29	257121.2	g2595803	3528	3819
29	257121.2	g1994549	3533	3860
29	257121.2	g613905	3177	3513
29	257121.2	5905841H1	3186	3500
29	257121.2	1444396H1	3186	3461
29	257121.2	g4187265	3195	3670
29	257121.2	2473816T6	3190	3808
29	257121.2	g4186245	3195	3664
29	257121.2	g3278632	3196	3616
29	257121.2	g1506304	3213	3436
29	257121.2	1658291H1	3211	3453
29	257121.2	g1994550	2249	2499
29	257121.2	3053733H1	2279	2581
29	257121.2	5641114H1	2298	2545
29	257121.2	995725R1	2300	2819
29	257121.2	995725H1	2300	2596
29	257121.2	2173193F6	2302	2653
29	257121.2	2173193H1	2302	2536
29	257121.2	3474978H1	2324	2601
29	257121.2	2106564H1	2329	2585
29	257121.2	5171463H1	2343	2629
29	257121.2	3449591H1	2355	2601
29	257121.2	2824470T6	2356	2941
29	257121.2	5546639H1	2387	2525
29	257121.2	5658068H1	2396	2642
29	257121.2	3771054H1	1386	1692
29	257121.2	2714558T6	1407	1749
29	257121.2	g2037431	1419	1693
29	257121.2	2436316H1	1459	1650
29	257121.2	g953893	1502	1633
29	257121.2	6075654H1	1487	1796
29	257121.2	g1055687	1504	1601
29	257121.2	g2034372	1557	1818
29	257121.2	1235661H1	1567	1836
29	257121.2	2824470F6	1573	1977
29	257121.2	2824470H1	1573	1809
29	257121.2	g4509712	1585	1995
29	257121.2	g3700825	1587	1927
29	257121.2	4876427H1	1618	1807



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SEQ ID NO:	Template ID	Component ID	Start	Stop
29	257121.2	3297894H1	1657	1904
29	257121.2	g3764184	1663	1795
29	257121.2	4306167H1	1689	1809
29	257121.2	44631976	1703	2175
29	257121.2	5984668H1	1802	2091
29	257121.2	5594456H1	1805	1931
29	257121.2	3202943H1	1818	2073
29	257121.2	309745H1	3396	3648
29	257121.2	4652103H1	3402	3556
29	257121.2	g3058761	3407	3849
29	257121.2	g3961167	3409	3844
29	257121.2	g3178632	3413	3849
29	257121.2	5645258H1	190	462
29	257121.2	g751765	209	449
29	257121.2	g751766	344	636
29	257121.2	2478708H1	363	607
29	257121.2	5544313H1	370	582
29	257121.2	1911275H1	460	725
29	257121.2	1437425F1	511	985
29	257121.2	1437426H1	511	734
29	257121.2	1437425H1	511	736
29	257121.2	3697654H1	541	823
29	257121.2	3333937H1	589	769
29	257121.2	2639996H1	706	952
29	257121.2	446319R6	788	1254
29	257121.2	446319H1	788	1039
29	257121.2	3068429F7	1	419
29	257121.2	3068429H1	1	291
29	257121.2	2821269H1	126	301
29	257121.2	3160523H1	3217	3501
29	257121.2	5024976H1	3222	3513
29	257121.2	3894771H1	3241	3546
29	257121.2	3360915H1	3240	3354
29	257121.2	1427954T6	3250	3809
29	257121.2	1741564T6	3251	3811
29	257121.2	1892219H1	3250	3516
29	257121.2	2173193T6	3255	3819
29	257121.2	1861145H1	3261	3586
29	257121.2	1531024H1	3769	3849
29	257121.2	2608136H1	3540	3797
29	257121.2	2608136T6	3533	3805
29	257121.2	2608136F6	3540	3853
29	257121.2	g1210010	3540	3850
29	257121.2	g2805071	3540	3827
29	257121.2	g2218792	3542	3849
29	257121.2	2721666H1	3563	3814
29	257121.2	4901093H1	3614	3850
29	257121.2	g3092920	3617	3818
29	257121.2	4347921H1	3623	3877
29	257121.2	3109370H1	3625	3723

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SEQ ID NO:	Template ID	Component ID	Start	Stop
29	257121.2	g1757345	3644	3855
29	257121.2	g12111126	3645	3857
29	257121.2	g1505472	3647	3864
29	257121.2	5565971H1	3650	3853
29	257121.2	4613032H1	3668	3856
29	257121.2	892266H1	3672	3830
29	257121.2	3729288H1	3676	3853
29	257121.2	g1813011	3677	3855
29	257121.2	g2063050	3680	3849
29	257121.2	3729209T1	3681	3810
29	257121.2	g2063735	3707	3849
29	257121.2	g2882056	3741	3849
29	257121.2	1547677H1	3769	3844
29	257121.2	6024576H1	2558	2843
29	257121.2	4909221H1	2607	2901
29	257121.2	1427954H1	2634	2884
29	257121.2	5170995H1	2648	2874
29	257121.2	1427954F6	2665	3120
29	257121.2	946401H1	2666	2926
29	257121.2	1374579H1	2670	2923
29	257121.2	2261955H1	2684	2948
29	257121.2	3094766H1	2691	2994
29	257121.2	2741005H1	2693	2978
29	257121.2	4635067H1	2704	2978
29	257121.2	1007503H1	2718	3048
29	257121.2	5284328H1	2720	2957
29	257121.2	5064384H1	2731	2941
29	257121.2	1672059H1	2742	2924
29	257121.2	1672008H1	2742	2975
29	257121.2	1672059F6	2742	3123
29	257121.2	2410045H1	2774	3011
37	84399.1	2520472H1	1	226
37	84399.1	g4148125	155	499
38	350044.1	3110061F7	1	276
38	350044.1	3110061H1	3	289
38	350044.1	4308349H1	158	426
38	350044.1	4308349F6	158	587
38	350044.1	5333549H1	185	413
38	350044.1	3399811H1	405	650
38	350044.1	2288313H1	496	629
38	350044.1	4637040H1	583	844
38	350044.1	4637040F6	582	916
38	350044.1	4308349T6	662	1020
38	350044.1	308559H1	664	884
38	350044.1	g1803082	832	926
38	350044.1	3977826H1	852	968
39	441329.2	g3751157	1	412
39	441329.2	g1470664	342	578
39	441329.2	g1395945	342	644
39	441329.2	4327736H1	355	622

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SEQ ID NO:	Template ID	Component ID	Start	Stop
39	441329.2	132848H1	360	540
39	441329.2	132849R6	360	805
39	441329.2	131890H1	360	563
39	441329.2	131890R6	360	802
39	441329.2	g1165579	361	542
39	441329.2	g1928191	371	650
39	441329.2	4204432F6	374	765
39	441329.2	g1734964	378	763
39	441329.2	3357071H1	701	979
39	441329.2	3357071F6	701	1118
39	441329.2	131890T6	1077	1235
39	441329.2	g1733361	1084	1235
39	441329.2	132849T6	1123	1235
39	441329.2	4204432T6	1128	1235
40	442401.2	3349655H1	1	327
40	442401.2	4309840H1	10	304
40	442401.2	4349106H1	25	238
40	442401.2	5043378H1	42	296
40	442401.2	4789236H1	44	123
40	442401.2	5320882H1	45	180
40	442401.2	2551237H1	64	319
40	442401.2	4664370H1	71	322
40	442401.2	3510753H1	75	389
40	442401.2	693783H1	80	286
40	442401.2	693783R6	83	554
40	442401.2	3865603H1	88	388
40	442401.2	2289862H1	86	326
40	442401.2	3681694H1	92	386
40	442401.2	693783T6	128	736
40	442401.2	519767H1	511	741
41	444933.2	3492265H1	7	310
41	444933.2	g1501708	7	263
41	444933.2	3155609H1	7	99
41	444933.2	g1474433	9	386
41	444933.2	3118539H1	9	310
41	444933.2	3295816H1	15	277
41	444933.2	1592931H1	29	229
41	444933.2	1592931F6	29	395
41	444933.2	1592931T6	43	579
41	444933.2	3436778H1	1	229
42	481129.4	999203H1	234	552
42	481129.4	999203T1	234	708
42	481129.4	g3278054	236	751
42	481129.4	3868296H1	240	560
42	481129.4	g4522433	240	752
42	481129.4	3137701H1	240	575
42	481129.4	g3417930	241	746
42	481129.4	3868754H1	240	570
42	481129.4	g4070375	243	752
42	481129.4	715742H1	248	618

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SEQ ID NO:	Template ID	Component ID	Start	Stop
42	481129.4	4950629H1	245	592
42	481129.4	g3644668	250	755
42	481129.4	1577433H1	250	516
42	481129.4	g3446453	265	747
42	481129.4	2055077H1	267	589
42	481129.4	g2288119	272	746
42	481129.4	3358220H1	293	617
42	481129.4	g3797800	295	747
42	481129.4	g2113046	296	749
42	481129.4	g2740428	301	747
42	481129.4	g2268545	312	747
42	481129.4	g4087282	314	750
42	481129.4	g2017050	320	622
42	481129.4	g1924476	320	647
42	481129.4	g3735602	323	748
42	481129.4	g4532931	324	747
42	481129.4	g3734942	324	751
42	481129.4	g2737174	325	747
42	481129.4	g3960674	330	751
42	481129.4	g2252043	328	746
42	481129.4	g3330470	342	747
42	481129.4	g3960381	342	755
42	481129.4	g4452123	351	746
42	481129.4	g3678389	352	747
42	481129.4	1632555H1	353	564
42	481129.4	1632539H1	353	571
42	481129.4	4228443H1	353	686
42	481129.4	g3108753	369	749
42	481129.4	g2319165	368	747
42	481129.4	g3755458	374	754
42	481129.4	g4086634	397	747
42	481129.4	g2322475	404	746
42	481129.4	g3750698	406	748
42	481129.4	g2021853	411	746
42	481129.4	3099690H1	423	747
42	481129.4	4125491H1	425	735
42	481129.4	5107680H1	443	746
42	481129.4	1253080F1	454	746
42	481129.4	2741014H1	456	747
42	481129.4	g4086632	472	747
42	481129.4	3381102H1	195	363
42	481129.4	3136744H1	132	344
42	481129.4	2485548H1	37	110
42	481129.4	3238530H1	36	320
42	481129.4	5332434H1	132	262
42	481129.4	1743373H1	37	342
42	481129.4	2398059H1	30	282
42	481129.4	5299970H1	36	291
42	481129.4	4857940H1	132	351
42	481129.4	982689H1	28	284

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SEQ ID NO:	Template ID	Component ID	Start	Stop
42	481129.4	1684768H1	34	296
42	481129.4	4901916H1	29	347
42	481129.4	4340959H1	25	317
42	481129.4	1275760H1	195	375
42	481129.4	g1975121	132	336
42	481129.4	534025H1	30	348
42	481129.4	3555252H1	24	317
42	481129.4	1504151H1	30	329
42	481129.4	4652858H1	195	379
42	481129.4	4638612H1	132	320
42	481129.4	4801179H1	30	310
42	481129.4	1970489H1	35	306
42	481129.4	2497883H1	30	272
42	481129.4	2557615H1	132	324
42	481129.4	4079990H1	195	386
42	481129.4	3555091H1	31	350
42	481129.4	g1635875	160	388
42	481129.4	3010470H1	132	368
42	481129.4	g1685660	488	846
42	481129.4	2716085H1	492	747
42	481129.4	g2669356	508	750
42	481129.4	g2035379	508	768
42	481129.4	g4330124	517	749
42	481129.4	g1125228	503	768
42	481129.4	g1237906	515	746
42	481129.4	1253080H1	547	746
42	481129.4	1924052H1	559	747
42	481129.4	1444584H1	557	747
42	481129.4	3089459H1	563	755
42	481129.4	1952233H1	596	746
42	481129.4	g2269237	609	746
42	481129.4	2058183R6	620	747
42	481129.4	2058183H1	620	747
42	481129.4	g3321468	627	746
42	481129.4	g2277314	629	747
42	481129.4	4363320H1	652	742
42	481129.4	g2752791	670	746
42	481129.4	3221190H1	49	104
42	481129.4	529956H1	197	303
42	481129.4	1781280H1	202	315
42	481129.4	586079H1	35	104
42	481129.4	644429H1	37	104
42	481129.4	4713384H1	32	97
42	481129.4	1434389H1	30	104
42	481129.4	2286414H1	30	104
42	481129.4	1648951H1	34	111
42	481129.4	3589355H1	36	111
42	481129.4	1916331H1	195	338
42	481129.4	880829H1	28	97
42	481129.4	2475170H1	195	350

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SEQ ID NO:	Template ID	Component ID	Start	Stop
42	481129.4	g1301383	263	352
42	481129.4	4612347H1	169	315
42	481129.4	116571H1	25	83
42	481129.4	g1023181	141	205
42	481129.4	835155H1	37	333
42	481129.4	4153116H1	132	329
42	481129.4	4300976H1	132	343
42	481129.4	962735H1	132	324
42	481129.4	4904613H2	132	339
42	481129.4	4904321H1	195	362
42	481129.4	4130260H1	132	334
42	481129.4	534025F1	1	184
42	481129.4	5165213H2	4	265
42	481129.4	g3085628	1	187
42	481129.4	g2836097	1	184
42	481129.4	g2397633	1	179
42	481129.4	g2669712	1	181
42	481129.4	811971T1	1	191
42	481129.4	811971H1	1	67
42	481129.4	g1578417	1	184
42	481129.4	g3016145	1	253
42	481129.4	g1425361	1	232
42	481129.4	g2237584	1	231
42	481129.4	g4072976	1	234
42	481129.4	g3076657	1	252
42	481129.4	3716227H1	1	125
42	481129.4	g2350483	1	232
42	481129.4	g1289724	1	184
42	481129.4	4830687H1	14	227
42	481129.4	1270745H1	1	82
42	481129.4	g3423624	1	187
42	481129.4	g1685572	1	232
42	481129.4	g2907402	1	79
42	481129.4	g2987865	1	235
42	481129.4	4664951H1	1	115
42	481129.4	g1114304	1	162
42	481129.4	1694416H1	1	78
42	481129.4	g4076242	1	234
42	481129.4	g2806310	1	181
42	481129.4	g3110520	1	231
42	481129.4	g3178699	1	235
42	481129.4	5691870H1	1	56
42	481129.4	g2408708	1	232
42	481129.4	g3070820	1	231
42	481129.4	g1194184	1	183
42	481129.4	g3400227	1	296
42	481129.4	g1080367	1	151
42	481129.4	g2768589	1	231
42	481129.4	g3804766	1	184
42	481129.4	g3190855	1	232

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SEQ ID NO:	Template ID	Component ID	Start	Stop
42	481129.4	g1087452	1	267
42	481129.4	4065091H1	1	169
42	481129.4	g2838959	1	236
42	481129.4	3091849H1	1	226
42	481129.4	g2263870	1	183
42	481129.4	g2279285	1	239
42	481129.4	2755370H1	1	184
42	481129.4	2757311H1	1	184
42	481129.4	863475H1	1	192
42	481129.4	g1689421	1	183
42	481129.4	5528923H1	19	211
42	481129.4	g1506021	1	184
42	481129.4	2467514H1	10	261
42	481129.4	2943819H1	15	312
42	481129.4	g1043884	16	196
42	481129.4	4461552H1	5	231
42	481129.4	g1281569	1	531
42	481129.4	g727471	1	234
42	481129.4	4889929H1	29	314
42	481129.4	4985113H1	29	354
42	481129.4	1488272H1	5	319
42	481129.4	2450834F6	6	558
42	481129.4	2450834H1	6	287
42	481129.4	g2027943	7	201
42	481129.4	2394311H2	8	123
42	481129.4	3372442H1	1	258
42	481129.4	g3307272	28	238
42	481129.4	3773060H1	38	366
42	481129.4	4125777H1	10	329
42	481129.4	5159564H1	12	302
42	481129.4	3233460H1	12	297
42	481129.4	5552762H1	12	315
42	481129.4	593632H1	12	123
42	481129.4	4513470H1	13	313
42	481129.4	5115162H1	44	356
42	481129.4	5039159H2	15	286
42	481129.4	2134140H1	15	328
42	481129.4	5943202H1	44	374
42	481129.4	027622R6	17	589
42	481129.4	027622H1	17	197
42	481129.4	3115953H1	46	372
42	481129.4	3489959H1	46	382
42	481129.4	5166761H1	44	322
42	481129.4	3626341H1	18	386
42	481129.4	3645933H1	48	327
42	481129.4	3159541H1	19	348
42	481129.4	2741058H1	52	282
42	481129.4	1475821H1	50	252
42	481129.4	4567661H1	22	350
42	481129.4	3601462H1	22	394

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SEQ ID NO:	Template ID	Component ID	Start	Stop
42	481129.4	4595577H1	53	283
42	481129.4	3819717H1	22	426
42	481129.4	4975524H1	22	359
42	481129.4	2084168H1	50	348
42	481129.4	5529123H1	18	212
42	481129.4	5700770H1	52	344
42	481129.4	5701471H1	52	355
42	481129.4	g3134982	45	234
42	481129.4	4979603H1	52	355
42	481129.4	3202264H1	23	321
42	481129.4	4938763H1	16	130
42	481129.4	4202906H1	8	274
42	481129.4	526404H1	22	322
42	481129.4	2665708H1	21	280
42	481129.4	2941705H1	22	327
42	481129.4	g1321143	11	346
42	481129.4	g2021854	22	337
42	481129.4	1833977H1	22	134
42	481129.4	2100632H1	12	285
42	481129.4	3675543H1	12	320
42	481129.4	3186510H1	13	400
42	481129.4	3229333H1	14	321
42	481129.4	4885571H1	19	306
42	481129.4	3074685H1	16	321
42	481129.4	3663968H1	16	329
42	481129.4	g2154555	1	29
42	481129.4	863475R1	1	192
42	481129.4	4230048H1	1	176
42	481129.4	3693558H1	18	328
42	481129.4	g1648658	1	23
42	481129.4	584205H1	18	309
42	481129.4	4227248H1	21	324
42	481129.4	g1043979	28	415
42	481129.4	g2142101	1	24
42	481129.4	4203722H1	22	321
42	481129.4	4174825H1	22	337
42	481129.4	5115519H1	23	321
42	481129.4	3133373H1	25	334
42	481129.4	1275760F6	1	147
42	481129.4	1275760F1	1	76
42	481129.4	2051350H1	27	350
42	481129.4	4341005H1	27	398
42	481129.4	3134845H1	1	260
42	481129.4	3484150H1	1	316
42	481129.4	4584348H1	1	279
42	481129.4	g2004902	1	283
42	481129.4	4374817H1	1	288
42	481129.4	g1967486	1	183
42	481129.4	4147580H1	1	267
42	481129.4	g1425470	1	78



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TABLE 4

SEQ ID NO:	Template ID	Component ID	Start	Stop
42	481129.4	4276424H1	1	301
42	481129.4	082654H1	15	171
42	481129.4	g3003216	1	306
42	481129.4	g1648659	1	183
42	481129.4	4563261H1	1	255
42	481129.4	4563351H1	12	136
42	481129.4	116571F1	1	233
42	481129.4	g4372584	1	234
42	481129.4	3994691H1	23	288
42	481129.4	g2437312	1	188
42	481129.4	136145F1	1	184
42	481129.4	2768229H1	1	256
42	481129.4	5852546H1	1	272
42	481129.4	5844492H1	6	279
42	481129.4	1727158H1	8	249
42	481129.4	1950091H1	8	264
42	481129.4	3204976H1	10	290
42	481129.4	4554858H1	10	275
42	481129.4	5840991H2	11	303
42	481129.4	5840890H2	11	314
42	481129.4	4559105H1	12	298
42	481129.4	871105H1	12	123
42	481129.4	4998348H1	12	293
42	481129.4	871105R1	12	123
42	481129.4	4770486H1	13	287
42	481129.4	3426271H1	14	295
42	481129.4	3273645H1	14	298
42	481129.4	4502129H1	14	202
42	481129.4	3403224H1	15	221
42	481129.4	1511018H1	15	89
42	481129.4	4502229H1	15	288
42	481129.4	4042436H1	8	283
42	481129.4	3845464H1	10	357
42	481129.4	3720453H1	12	340
42	481129.4	1998071H1	22	123
42	481129.4	5835243H1	22	290
42	481129.4	3801167H1	22	236
42	481129.4	746353H1	12	243
42	481129.4	g1685558	13	441
42	481129.4	2537659H1	15	303
42	481129.4	1728205H1	13	209
42	481129.4	2674637H1	15	271
42	481129.4	4855434H1	14	293
42	481129.4	2943583H2	15	322
42	481129.4	116571R1	1	182
42	481129.4	g1320320	1	363
42	481129.4	2801342H1	5	264
42	481129.4	5528955H1	21	208
42	481129.4	3618142H1	24	377
42	481129.4	122118H1	17	164

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TABLE 4

SEQ ID NO:	Template ID	Component ID	Start	Stop
42	481129.4	3580438H1	69	320
42	481129.4	g1638276	25	435
42	481129.4	833841T1	40	708
42	481129.4	3997421H1	26	345
42	481129.4	4110340H1	56	374
42	481129.4	2403135H1	58	320
42	481129.4	g1969685	58	472
42	481129.4	3026745H1	57	366
42	481129.4	833841H1	40	406
42	481129.4	5681519H1	42	198
42	481129.4	3987429H1	58	376
42	481129.4	5177261H2	70	330
42	481129.4	880829R1	29	768
42	481129.4	g1954123	46	346
42	481129.4	755972H1	59	323
42	481129.4	755972R1	59	709
42	481129.4	3944829H1	77	366
42	481129.4	1546083H1	58	262
42	481129.4	g1496701	1	184
42	481129.4	962735R2	78	716
42	481129.4	2561965H1	32	359
42	481129.4	g2008330	80	340
42	481129.4	881179T1	30	687
42	481129.4	534025R1	32	572
42	481129.4	871105T1	54	123
42	481129.4	g1956736	64	347
42	481129.4	4800988H1	83	224
42	481129.4	g1685765	83	403
42	481129.4	1230023H1	32	172
42	481129.4	1805729H1	27	189
42	481129.4	1320892H1	38	321
42	481129.4	1599902H1	38	290
42	481129.4	4588734H1	85	374
42	481129.4	g1955622	70	445
42	481129.4	3517903H1	40	255
42	481129.4	136145R1	69	590
42	481129.4	4979660H1	40	354
42	481129.4	1741320H1	42	338
42	481129.4	5117019H1	88	386
42	481129.4	g1924567	88	536
42	481129.4	2343536H1	89	355
42	481129.4	4536803H1	90	398
42	481129.4	g2158823	96	655
42	481129.4	g1958858	97	676
42	481129.4	g828123	93	501
42	481129.4	g3988556	97	621
42	481129.4	g3096874	99	539
42	481129.4	g3178165	107	566
42	481129.4	g4079967	110	541
42	481129.4	465693H1	66	199

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TABLE 4

SEQ ID NO:	Template ID	Component ID	Start	Stop
42	481129.4	g3003210	111	467
42	481129.4	5265746H1	120	374
42	481129.4	966316H1	118	374
42	481129.4	1458085H1	122	396
42	481129.4	1457484H1	122	305
42	481129.4	2109149H1	122	442
42	481129.4	2109149R6	123	704
42	481129.4	g1243536	77	215
42	481129.4	958148H1	122	431
42	481129.4	5020591T1	124	705
42	481129.4	g1087561	133	459
42	481129.4	g1958920	135	700
42	481129.4	g1147415	139	444
42	481129.4	g3890878	103	203
42	481129.4	2401220H1	144	390
42	481129.4	3223013H1	149	494
42	481129.4	1680890H1	149	361
42	481129.4	g1298313	155	667
42	481129.4	828345H1	143	226
42	481129.4	3441046H1	159	442
42	481129.4	2109149T6	163	707
42	481129.4	2004957H1	176	484
42	481129.4	6063286H1	180	511
42	481129.4	5020591H1	179	501
42	481129.4	2450834T6	182	707
42	481129.4	027622T6	183	830
42	481129.4	5597771H1	189	470
42	481129.4	821831T6	192	705
42	481129.4	821831R1	192	746
42	481129.4	821831R6	192	746
42	481129.4	821831F1	192	757
42	481129.4	g2252207	199	748
42	481129.4	1457484R1	199	746
42	481129.4	g3736470	203	746
42	481129.4	g1295465	204	757
42	481129.4	5185783H1	205	496
42	481129.4	g2115256	211	352
42	481129.4	4085165H1	215	541
42	481129.4	g1577977	221	716
42	481129.4	g3593921	222	750
42	481129.4	g3429019	222	747
42	481129.4	5221813H2	225	517
42	481129.4	5863560H1	232	539
42	481129.4	999208H1	234	544
42	481129.4	999203R1	234	750
42	481129.4	g3649202	235	753
42	481129.4	g2265393	234	751
42	481129.4	4049323H1	235	584
43	481999.1	1255239H1	838	1104
43	481999.1	4913234H1	397	512

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TABLE 4

SEQ ID NO:	Template ID	Component ID	Start	Stop
43	481999.1	1724376H1	864	932
43	481999.1	1509213H1	819	1012
43	481999.1	1849705H1	281	501
43	481999.1	1509213F6	819	1104
43	481999.1	1476972F6	260	574
43	481999.1	g2195301	866	1104
43	481999.1	g985997	864	941
43	481999.1	880602R1	397	512
43	481999.1	g1980533	1	283
43	481999.1	g2195281	987	1104
43	481999.1	5526658H1	780	991
43	481999.1	880602H1	394	457
43	481999.1	1255206H1	838	1104
43	481999.1	1509479F6	819	1104
43	481999.1	3457008H1	397	481
43	481999.1	3641330H1	723	1014
43	481999.1	4181741H1	717	799
43	481999.1	3384087F6	604	857
43	481999.1	4760678H1	1	286
43	481999.1	1509221H1	819	1006
43	481999.1	1509455H1	819	997
43	481999.1	2603491H1	880	1104
43	481999.1	170070H1	797	982
43	481999.1	3427420F6	521	604
43	481999.1	4760678F6	1	499
43	481999.1	4913234F6	235	670
43	481999.1	4072789H1	237	499
43	481999.1	1524891F6	269	890
43	481999.1	398973R1	305	830
43	481999.1	4972169H1	420	695
43	481999.1	4357304H1	435	710
43	481999.1	4181741F6	718	1107
43	481999.1	3123901F6	380	434
43	481999.1	5585642H1	650	868
43	481999.1	1724376F6	864	941
43	481999.1	2938884H1	864	941
43	481999.1	3369038H1	429	706
43	481999.1	g1148977	1	387
43	481999.1	g1971119	842	1104
43	481999.1	g1761297	704	1005
43	481999.1	1476972H1	260	455
43	481999.1	4211180H1	642	873
43	481999.1	g3665013	371	434
43	481999.1	5153580H1	813	1066
43	481999.1	g2141617	440	513
43	481999.1	1509479H1	819	997
46	338992.1	5510632H1	1	202
46	338992.1	4782751F6	127	187
46	338992.1	4782751H1	127	372
46	338992.1	2987413H1	192	478

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TABLE 4

SEQ ID NO:	Template ID	Component ID	Start	Stop
46	338992.1	6064208H1	204	465
46	338992.1	g1984325	272	505
46	338992.1	g3239921	294	663
46	338992.1	g2026718	300	591
46	338992.1	g2816869	321	569
46	338992.1	4760384H1	492	580
46	338992.1	4760384F6	492	965
46	338992.1	1305496F6	802	1270
46	338992.1	1305496H1	802	945
46	338992.1	3617505H1	823	1024
46	338992.1	g875546	878	1132
46	338992.1	g669800	877	1199
46	338992.1	g874649	878	1180
46	338992.1	g771213	1011	1062
46	338992.1	3211592H1	1056	1292
46	338992.1	093259H1	1081	1322
46	338992.1	926880H1	1123	1363
46	338992.1	1366443H1	1137	1385
46	338992.1	1366443R6	1137	1466
46	338992.1	4960778H1	1160	1431
46	338992.1	4625867H1	1224	1487
46	338992.1	3383444H1	1259	1504
46	338992.1	5640688H1	1417	1657
46	338992.1	4030718H1	1568	1830
46	338992.1	4030718F6	1568	1945
46	338992.1	2742284H1	1620	1870
51	206603.1	g3278540	1	465
51	206603.1	g4390504	25	492
51	206603.1	g3870096	41	504
51	206603.1	g1109407	277	604
51	206603.1	5631413H1	367	608
51	206603.1	5631413F6	367	812
51	206603.1	1992082H1	531	726
51	206603.1	6092061H1	626	906
52	435694.2	4936540H1	1112	1364
52	435694.2	5021371H1	1139	1417
52	435694.2	3726226H1	1155	1450
52	435694.2	g2015121	1170	1366
52	435694.2	456419H1	1185	1425
52	435694.2	461227R6	1185	1685
52	435694.2	456301H1	1185	1433
52	435694.2	461227H1	1185	1446
52	435694.2	460920H1	1185	1432
52	435694.2	461106H1	1185	1440
52	435694.2	457987H1	1185	1424
52	435694.2	458221H1	1185	1435
52	435694.2	454834R1	1185	1705
52	435694.2	461049H1	1185	1425
52	435694.2	454834H1	1185	1424
52	435694.2	4957324H1	266	486

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TABLE 4

SEQ ID NO:	Template ID	Component ID	Start	Stop
52	435694.2	3390485H1	267	486
52	435694.2	1437407H1	243	486
52	435694.2	5902323H1	243	486
52	435694.2	3676724H1	271	486
52	435694.2	1437407F6	243	486
52	435694.2	1436837H1	243	480
52	435694.2	5900454H1	243	486
52	435694.2	2706491H1	270	566
52	435694.2	2837043H1	244	486
52	435694.2	3165547H1	246	486
52	435694.2	5903233H1	247	568
52	435694.2	3687630H1	251	565
52	435694.2	4268779H1	252	486
52	435694.2	3460048H1	251	487
52	435694.2	3077826H1	253	501
52	435694.2	4121058H1	271	486
52	435694.2	4900932H1	273	562
52	435694.2	3615215F6	280	731
52	435694.2	832322H1	252	486
52	435694.2	3615215H1	280	588
52	435694.2	2406515H1	254	486
52	435694.2	3141771H1	327	608
52	435694.2	3615215T6	351	720
52	435694.2	g1774842	351	728
52	435694.2	3932194H1	351	486
52	435694.2	4003539H1	351	589
52	435694.2	g587196	351	593
52	435694.2	1285417F6	400	708
52	435694.2	1285417H1	400	637
52	435694.2	2763950H1	502	762
52	435694.2	2415062H1	512	710
52	435694.2	2415070H1	512	702
52	435694.2	3860483H1	517	813
52	435694.2	g1766458	520	850
52	435694.2	g3076400	519	940
52	435694.2	823127H1	556	831
52	435694.2	g3190613	562	745
52	435694.2	2926224H2	688	999
52	435694.2	2708490H1	690	942
52	435694.2	g4311248	702	1175
52	435694.2	g4389608	707	1164
52	435694.2	g3700432	738	1174
52	435694.2	g1773867	738	1172
52	435694.2	1285417T6	758	904
52	435694.2	g4111706	797	1172
52	435694.2	774013H1	825	1036
52	435694.2	1454843H1	899	1163
52	435694.2	1450887F1	899	1172
52	435694.2	3742523H1	896	1163
52	435694.2	1450887H1	899	1152

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TABLE 4

SEQ ID NO:	Template ID	Component ID	Start	Stop
52	435694.2	3742566H1	899	1163
52	435694.2	1454843T6	913	1128
52	435694.2	4710458H1	961	1249
52	435694.2	4977443H1	1005	1268
52	435694.2	3237593H1	1060	1304
52	435694.2	4705379T6	1071	1144
52	435694.2	4853112H1	1100	1295
52	435694.2	2666646T6	1628	2175
52	435694.2	2959842H1	1635	1861
52	435694.2	2963934T6	1633	2162
52	435694.2	3639070H1	1655	1967
52	435694.2	4322530H1	1660	1782
52	435694.2	5062716H1	1673	1795
52	435694.2	1710033H1	1703	1959
52	435694.2	5021371T1	1715	2162
52	435694.2	3894718H1	1752	1968
52	435694.2	2151922H1	1765	2018
52	435694.2	g1645818	1810	2162
52	435694.2	2323908H1	1816	1878
52	435694.2	g4070415	1817	2162
52	435694.2	g4175351	1821	2162
52	435694.2	g3595864	1824	2162
52	435694.2	g3434496	1843	2162
52	435694.2	g1775010	1845	2162
52	435694.2	g1645819	1872	2162
52	435694.2	g1164565	1885	2262
52	435694.2	4701327H1	1906	2173
52	435694.2	g1766363	1910	2162
52	435694.2	2253564R6	1939	2171
52	435694.2	2253564H1	1939	2195
52	435694.2	4540530H1	1951	2181
52	435694.2	4540144H1	1953	2199
52	435694.2	1833058H1	1959	2171
52	435694.2	g3003709	1979	2171
52	435694.2	g3959558	1980	2162
52	435694.2	g2358889	2057	2171
52	435694.2	g4264824	2059	2162
52	435694.2	474448H1	2096	2171
52	435694.2	4615243H1	1	250
52	435694.2	2533844H1	21	246
52	435694.2	g3154852	56	491
52	435694.2	4270549H1	86	338
52	435694.2	3330903H1	114	331
52	435694.2	4315068H1	219	423
52	435694.2	4315053H1	221	486
52	435694.2	4706408H1	242	486
52	435694.2	1436837F1	243	695
52	435694.2	5900458H1	243	486
52	435694.2	5903501H1	242	486
52	435694.2	5903201H1	243	486

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TABLE 4

SEQ ID NO:	Template ID	Component ID	Start	Stop
52	435694.2	5426328H1	1199	1361
52	435694.2	g1989927	1223	1525
52	435694.2	g1989319	1223	1632
52	435694.2	g572779	1276	1621
52	435694.2	4611420H1	1351	1610
52	435694.2	4243682H1	1370	1625
52	435694.2	5868132H1	1375	1642
52	435694.2	792533H1	1412	1646
52	435694.2	g1775555	1428	1811
52	435694.2	1469570H1	1472	1684
52	435694.2	1469570F6	1472	1787
52	435694.2	2461531H1	1485	1710
52	435694.2	g2220895	1488	1893
52	435694.2	g1196070	1498	1783
52	435694.2	3561975H1	1527	1843
52	435694.2	1830391H1	1583	1847



Table 5

Program	Description	Reference	Parameter Threshold
ABI FACTURA	A program that removes vector sequences and masks ambiguous bases in nucleic acid sequences.	PE Biosystems, Foster City, CA.	
ABI/PARACHEL FDF	A Fast Data Finder useful in comparing and annotating amino acid or nucleic acid sequences.	PE Biosystems, Foster City, CA; Paracel Inc., Pasadena, CA.	Mismatch <50%
ABI AutoAssembler	A program that assembles nucleic acid sequences.	PE Biosystems, Foster City, CA.	
BLAST	A Basic Local Alignment Search Tool useful in sequence similarity search for amino acid and nucleic acid sequences. BLAST includes five functions: blastp, blastn, blastx, tblastn, and tblastx.	Altschul, S.F. et al. (1990) J. Mol. Biol. 215:403-410; Altschul, S.F. et al. (1997) Nucleic Acids Res. 25:3389-3402.	ESTs: Probability value= 1.0E-8 or less Full Length sequences: Probability value= 1.0E-10 or less
FASTA	A Pearson and Lipman algorithm that searches for similarity between a query sequence and a group of sequences of the same type. FASTA comprises at least five functions: fasta, fasta, fastx, fasty, and search.	Pearson, W.R. and D.J. Lipman (1988) Proc. Natl. Acad. Sci. USA 85:2444-2448; Pearson, W.R. (1990) Methods Enzymol. 183:63-98; and Smith, T.F. and M.S. Waterman (1981) Adv. Appl. Math. 2:482-489.	ESTs: fasta E value=1.0E-6 Assembled ESTs: fasta Identity= 95% or greater and Match length=200 bases or greater; fastx E value=1.0E-8 or less Full Length sequences: fastx score=100 or greater
BLIMPS	A BLOCKS IMProved Searcher that matches a sequence against those in BLOCKS, PRINTS, DOMO, PRODOM, and PFAM databases to search for gene families, sequence homology, and structural fingerprint regions.	Henikoff, S. and J.G. Henikoff (1991) Nucleic Acids Res. 19:6565-6572; Henikoff, J.G. and S. Henikoff (1996) Methods Enzymol. 266:88-105; and Attwood, T.K. et al. (1997) J. Chem. Inf. Comput. Sci. 37:417-424.	Score=1000 or greater; Ratio of Score/Strength = 0.75 or larger; and, if applicable, Probability value= 1.0E-3 or less
HMMER	An algorithm for searching a query sequence against hidden Markov model (HMM)-based databases of protein family consensus sequences, such as PFAM.	Krogh, A. et al. (1994) J. Mol. Biol. 235:1501-1531; Sonnhammer, E.L.L. et al. (1988) Nucleic Acids Res. 26:320-322.	Score=10-50 bits for PFAM hits, depending on individual protein families

Table 5 (cont.)

Program	Description	Reference	Parameter Threshold
ProfileScan	An algorithm that searches for structural and sequence motifs in protein sequences that match sequence patterns defined in Prosite.	Gribskov, M. et al. (1988) CABIOS 4:61-66; Gribskov, M. et al. (1989) Methods Enzymol. 183:146-159; Bairoch, A. et al. (1997) Nucleic Acids Res. 25:217-221.	Normalized quality scores:GCC-specified "HIGH" value for that particular Prosite motif. Generally, score=1.4-2.1.
Phred	A base-calling algorithm that examines automated sequencer traces with high sensitivity and probability.	Ewing, B. et al. (1998) Genome Res. 8:175-185; Ewing, B. and P. Green (1998) Genome Res. 8:186-194.	
Phrap	A Phils Revised Assembly Program including SWAT and CrossMatch, programs based on efficient implementation of the Smith-Waterman algorithm, useful in searching sequence homology and assembling DNA sequences.	Smith, T.F. and M.S. Waterman (1981) Adv. Appl. Math. 2:482-489; Smith, T.F. and M.S. Waterman (1981) J. Mol. Biol. 147:195-197; and Green, P., University of Washington, Seattle, WA.	Score= 120 or greater; Match length= 56 or greater
Consed	A graphical tool for viewing and editing Phrap assemblies.	Gordon, D. et al. (1998) Genome Res. 8:195-202.	
SPScan	A weight matrix analysis program that scans protein sequences for the presence of secretory signal peptides.	Nielson, H. et al. (1997) Protein Engineering 10:1-6; Claverie, J.M. and S. Audic (1997) CABIOS 12:431-439.	Score=3.5 or greater
Motifs	A program that searches amino acid sequences for patterns that matched those defined in Prosite.	Bairoch, A. et al. (1997) Nucleic Acids Res. 25:217-221; Wisconsin Package Program Manual, version 9, page M51-59, Genetics Computer Group, Madison, WI.	

# CLAIMS

## What is claimed is:

1. An isolated polynucleotide comprising a polynucleotide sequence selected from the group  
5 consisting of:
  - a) a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52,
  - b) a naturally occurring polynucleotide sequence having at least 90% sequence identity to a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52,
  - c) a polynucleotide sequence complementary to a),
  - 10 d) a polynucleotide sequence complementary to b), and
  - e) an RNA equivalent of a) through d).
2. An isolated polynucleotide of claim 1, comprising a polynucleotide sequence selected from the group consisting of SEQ ID NO:1-52.
- 15 3. An isolated polynucleotide comprising at least 60 contiguous nucleotides of a polynucleotide of claim 1.
4. A composition for the detection of expression of diagnostic and therapeutic polynucleotides  
20 comprising at least one of the polynucleotides of claim 1 and a detectable label.
5. A method for detecting a target polynucleotide in a sample, said target polynucleotide having a sequence of a polynucleotide of claim 1, the method comprising:
  - a) amplifying said target polynucleotide or fragment thereof using polymerase chain reaction
  - 25 amplification, and
  - b) detecting the presence or absence of said amplified target polynucleotide or fragment thereof, and, optionally, if present, the amount thereof.
6. A method for detecting a target polynucleotide in a sample, said target polynucleotide  
30 comprising a sequence of a polynucleotide of claim 1, the method comprising:
  - a) hybridizing the sample with a probe comprising at least 20 contiguous nucleotides comprising a sequence complementary to said target polynucleotide in the sample, and which probe specifically hybridizes to said target polynucleotide, under conditions whereby a hybridization complex is formed between said probe and said target polynucleotide, and
  - 35 b) detecting the presence or absence of said hybridization complex, and, optionally, if present,

the amount thereof.

7. A method of claim 5, wherein the probe comprises at least 30 contiguous nucleotides.
- 5       8. A method of claim 5, wherein the probe comprises at least 60 contiguous nucleotides.
9. A recombinant polynucleotide comprising a promoter sequence operably linked to a polynucleotide of claim 1.
- 10       10. A cell transformed with a recombinant polynucleotide of claim 9.
11. A transgenic organism comprising a recombinant polynucleotide of claim 9.
12. A method for producing a diagnostic and therapeutic polypeptide, the method comprising:  
15       a) culturing a cell under conditions suitable for expression of the diagnostic and therapeutic polypeptide, wherein said cell is transformed with a recombinant polynucleotide of claim 9, and  
       b) recovering the diagnostic and therapeutic polypeptide so expressed.
13. A purified diagnostic and therapeutic polypeptide (DITHP) encoded by at least one of the  
20       polynucleotides of claim 2.
14. An isolated antibody which specifically binds to a diagnostic and therapeutic polypeptide of claim 13.
- 25       15. A method of identifying a test compound which specifically binds to the diagnostic and therapeutic polypeptide of claim 13, the method comprising the steps of:  
       a) providing a test compound;  
       b) combining the diagnostic and therapeutic polypeptide with the test compound for a sufficient time and under suitable conditions for binding; and  
30       c) detecting binding of the diagnostic and therapeutic polypeptide to the test compound, thereby identifying the test compound which specifically binds the diagnostic and therapeutic polypeptide.
16. A microarray wherein at least one element of the microarray is a polynucleotide of claim 3.

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17. A method for generating a transcript image of a sample which contains polynucleotides, the method comprising the steps of:

- a) labeling the polynucleotides of the sample,
- b) contacting the elements of the microarray of claim 16 with the labeled polynucleotides of the
- 5 sample under conditions suitable for the formation of a hybridization complex, and
- c) quantifying the expression of the polynucleotides in the sample.

18. A method for screening a compound for effectiveness in altering expression of a target polynucleotide, wherein said target polynucleotide comprises a polynucleotide sequence of claim 1, the

10 method comprising:

- a) exposing a sample comprising the target polynucleotide to a compound, and
- b) detecting altered expression of the target polynucleotide.

19. A method of claim 6 for toxicity testing of a compound, further comprising:

- 15 c) comparing the presence, absence or amount of said target polynucleotide in a first biological sample and a second biological sample, wherein said first biological sample has been contacted with said compound, and said second sample is a control, whereby a change in presence, absence or amount of said target polynucleotide in said first sample, as compared with said second sample, is indicative of toxic response to said compound.

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SEQUENCE LISTING

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      BRATCHER, Shawn R.
      DUFOUR, Gerard E.
      COHEN, Howard J.
      ROSEN, Bruce
      CHALUP, Michael S.
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      JONES, Annisa L.
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<223> a, t, c, g, or other

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```

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 <223> Incyte ID No: 394637.1.j

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&lt;211&gt; 1307

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&lt;223&gt; Incyte ID No: 222429.3

&lt;400&gt; 18

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&lt;213&gt; Homo sapiens

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&lt;223&gt; Incyte ID No: 366739.2

&lt;220&gt;

&lt;221&gt; unsure

&lt;222&gt; 311

&lt;223&gt; a, t, c, g, or other

&lt;400&gt; 19

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&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;220&gt;

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&lt;223&gt; Incyte ID No: 474635.6

&lt;220&gt;

&lt;221&gt; unsure

&lt;222&gt; 2063-2064, 2068, 2070, 2078-2079

&lt;223&gt; a, t, c, g, or other

&lt;400&gt; 20

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&lt;223&gt; Incyte ID No: 228470.1.j

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&lt;221&gt; unsure

&lt;222&gt; 110, 114, 159, 163

&lt;223&gt; a, t, c, g, or other

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&lt;220&gt;

&lt;221&gt; unsure

&lt;222&gt; 2926, 2938, 2941, 3008, 3052

&lt;223&gt; a, t, c, g, or other

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<223> a, t, c, g, or other

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 <222> 34, 418  
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<210> 27

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 <212> DNA  
 <213> Homo sapiens

<220>  
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<210> 29
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<213> Homo sapiens

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<223> a, t, c, g, or other

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<212> DNA
<213> Homo sapiens

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<220>
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<211> 514
<212> DNA
<213> Homo sapiens

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<220>
<221> misc_feature
<223> Incyte ID No: 442085.1.j

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 <221> unsure  
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 <212> DNA  
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<220>  
 <221> unsure  
 <222> 1284, 1412  
 <223> a, t, c, g, or other

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<212> DNA
<213> Homo sapiens

<220>
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<223> Incyte ID No: 430569.2.j

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<212> DNA
<213> Homo sapiens

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<211> 517
<212> DNA
<213> Homo sapiens

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<223> Incyte ID No: 445198.1.j

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<221> unsure
<222> 48-49
<223> a, t, c, g, or other

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 <221> unsure  
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 <223> a, t, c, g, or other

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<220>  
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 <223> Incyte ID No: 350044.1

<220>  
 <221> unsure  
 <222> 775  
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 tgcagcgaat gccagaaggc otttattaga aagtctcacc tggttcatac ccagaaaatc 960  
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<210> 39  
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 <212> DNA  
 <213> Homo sapiens

<220>  
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 <223> Incyte ID No: 441329.2

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<400> 39
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tgggacctga teetccaagc tggaggcctg aaaactcaag ggcaggcgcc ttggtccagcg 240
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<210> 40
<211> 730
<212> DNA
<213> Homo sapiens

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<220>
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<223> Incyte ID No: 442401.2

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<220>
<221> unsure
<222> 631
<223> a, t, c, g, or other

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agaacctctg ttactctgtg accgcgagcg accgggagat ccgtagctca gacgccagga 180
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catagaatto tctcgggagg agtgggaaaca cctggactca gatcagaagc ttttatattg 300
ggaatgtgat ttagaagaact acggaaacct ggtctctctg ggtctcgtg tctctaaccc 360
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<210> 41
<211> 575
<212> DNA
<213> Homo sapiens

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<220>
<221> misc_feature
<223> Incyte ID No: 444933.2

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<400> 41
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caggaactct cacacaaga ggttgagtct cctcgggttg ccttcagggg aatgtcatcc 180
cggtctagaa gactgtcaga aggtgtcag gcttctcaga actttgctt ccagcagaa 240
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tattctggctt caaagccaga tgtgattacc ttactggagc aaggggaaaga gccttgcgtg 480
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<210> 42
<211> 734
<212> DNA
<213> Homo sapiens

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<220>
<221> misc_feature
<223> Incyte ID No: 481129.4

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aggttggcga agagacggag tttcgtcatg ttggccaggc ccatttgaga tctttgaaga 180
talctctcaac gtgaggctct gctgccatga agtggaagt taagtgcctgg aacggcgctgg 240
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tggaggggca tcttgagact ccttctcat gctggcgccg atggctgctg ggacacgcgc 540
ccctgagctg caacaagggt gaaacaaggc ctggagctgc gtttgttttg ccatcactat 600
gttgacactt ttatccaata agtgaaaact cattaaacta ctcaaatctt gctggaggcc 660
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ctaaatgaat aaac 734

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<210> 43
<211> 1104
<212> DNA
<213> Homo sapiens

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<220>
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<223> Incyte ID No: 481999.1

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1074
<223> a, t, c, g, or other

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atattcagtt aggtgaaaga ngtgctcttc aagccatag actagatgag atcacgggtg 240
aatatgtaca gacaggggag aagaaaagtga ggaccatcaa gtgataggag accaaagngt 300
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agatgtgatg ctggagaact acatgaacct ggcctctgtg gaatgggaaa tacaacctag 540
aaccacacgg tcatcacttc agcagggttt ttgaaagaat caaatattca gtgggataca 600
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<210> 44

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<211> 665
<212> DNA
<213> Homo sapiens

<220>
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<223> Incyte ID No: 233814.1.j

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tacgagtcce tgaggatctg tgggtgtgag ccagccgcgt gcccttgggc aacggcgctg 180
ccggacagaa ttggcggttg ctgacgcctg gaaattcccc tgaagggtgga gcaccacca 240
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cgtgtttacc atgcagctgg ccaagacaag gctgcagaac cagcagaaag gccagcgctg 480
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<210> 45
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<213> Homo sapiens

<220>
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gtagagcaca gagttagagaa caactggcag cttgaaaaa acaccaagaa gaagaaatcg 360
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<210> 46
<211> 1935
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Incyte ID No: 338992.1

<220>
<221> unsure
<222> 52, 55, 83, 88, 96, 208, 511
<223> a, t, c, g, or other

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<210> 47
<211> 1709
<212> DNA
<213> Homo sapiens

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<210> 48
<211> 766

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<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Incyte ID No: 246727.5..j

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<213> Homo sapiens

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<220>
<221> unsure
<222> 889, 917, 1054, 1098, 1122
<223> a, t, c, g, or other

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<210> 50
<211> 558
<212> DNA
<213> Homo sapiens

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<220>
<221> misc_feature
<223> Incyte ID No: 441779.1.j

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<212> DNA
<213> Homo sapiens

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<223> a, t, c, g, or other

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WO 00/73509

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<220>
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<223> a, t, c, g, or other

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